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[Reprinted from THE JOURNAL OF BIOLOGICAL CHEMISTRY, February, 1938, Vol. 122, No. 3, pp. 661-664]

CONVERSION OF URONIC ACIDS INTO CORRESPONDING HEXOSES

IV. CATALYTIC REDUCTION OF THE METHYL ESTER OF DIACETONE *d*-GALACTURONIC ACID

BY P. A. LEVENE AND CLARENCE C. CHRISTMAN

(*From the Laboratories of The Rockefeller Institute for Medical Research*)

(Received for publication, November 20, 1937)

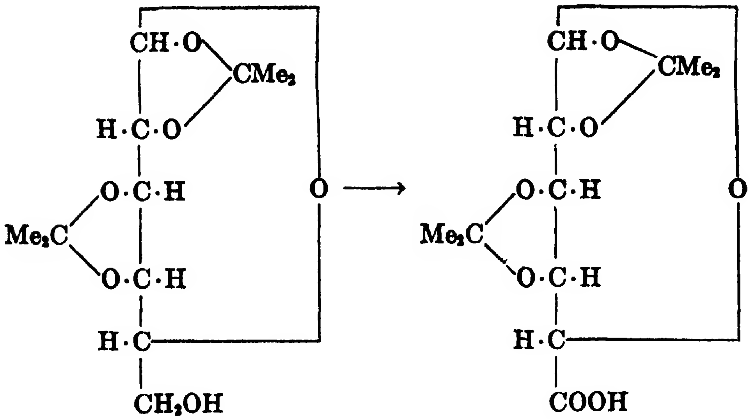
In previous communications[1] we have described the catalytic reduction of the methyl ester of 2,3,4-trimethyl α-methyl-*d*-galacturonide and of the methyl ester of 2,3,4-triacetyl α-methyl-*d*-galacturonide. In the case of simple uronic acids the conversion of the resulting glycosides into the free sugars is a simple procedure.

The situation is different in the case of aldobionic acids for the reason that the removal of the methoxyl group from carbon atom (1) of the resulting disaccharide glycoside requires treatment often sufficiently drastic to cleave the disaccharide linkage. It was thought that the acetone derivatives of uronic acids might retain the acetone residue during the process of reduction and thus might lead to acetone hexoses, which then could be converted readily into the corresponding free sugars. This expectation was actually realized.

The methyl ester of diacetone *d*-galacturonic acid was reduced to diacetone *d*-galactose under the same conditions as previously described. The stages leading from diacetone *d*-galactose back to the same substance are given in the accompanying formulæ.

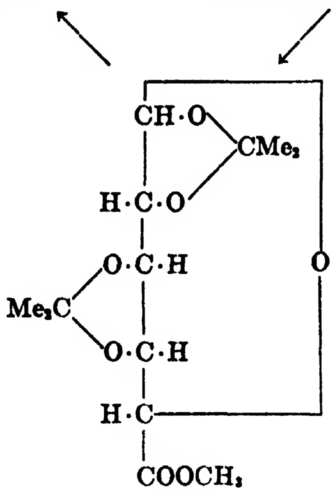
This method now is being applied to the conversion of certain free aldobionic acids into the corresponding disaccharides.

[1] Levene, P. A., Tipson, R. S., and Kreider, L. C., *J. Biol. Chem.*, **122**, 199 (1937-38). Levene, P. A., and Christman, C. C., *J. Biol. Chem.*, **122**, 203 (1937-38).



Diacetone *d*-galactose

Diacetone *d*-galacturonic acid



Methyl ester of diacetone *d*-galacturonic acid

EXPERIMENTAL

Attempted Catalytic Reduction of Diacetone d-Galactose—5 gm. of copper chromite catalyst were added to a solution of 5 gm. of diacetone *d*-galactose ($n_D^{25} = 1.4653$) in 100 cc. of absolute methanol. This mixture was placed in a high pressure reduction apparatus and a hydrogen pressure of 3000 pounds per sq. inch was applied. The temperature was now slowly increased to 175°. At this temperature the pressure rose to 4300 pounds per sq. inch. After the reaction had proceeded during 5 hours at this temperature, the whole apparatus was allowed to cool for 18 hours.

The reaction mixture was removed, some charcoal added, and

the catalyst separated by filtration. The filtrate was concentrated to dryness and the resulting sirup distilled under a high vacuum, giving three fractions all having a refractive index of $n_D^{25} = 1.4653$. Yield 4.8 gm. The material had the following specific rotation

$$[\alpha]_D^{25} = \frac{-6.67° \times 100}{2 \times 5.800} = -57.5° \text{ (in chloroform)}$$

and was therefore unchanged diacetone *d*-galactose.

Preparation of Methyl Ester of Diacetone d-Galacturonic Acid—10 gm. of dry diacetone galacturonic acid[2] (m.p. 156°) were slowly added to a cold solution of 2 gm. of diazomethane in 100 cc. of dry ether. The reaction was instantaneous and was accompanied by the rapid evolution of nitrogen. The mixture, after standing overnight at room temperature, still contained diazomethane, as indicated by the faint yellow color.

The solution was filtered and the filtrate evaporated under diminished pressure to a thick sirup which could not be induced to crystallize. It was therefore distilled under diminished pressure. The whole of the substance boiled at 133° (bath temperature) and 0.17 mm. pressure. Yield 9.7 gm. Four fractions were collected, each of which had a refractive index of $n_D^{25} = 1.4622$. The substance had the following specific rotation.

$$[\alpha]_D^{25} = \frac{-9.72° \times 100}{2 \times 5.200} = -93.4° \text{ (in chloroform)}$$

It is soluble in acetone, chloroform, benzene, heptane, ethyl and methyl alcohols, and ether but is practically insoluble in pentane or water.

The composition of the substance agreed with that for the methyl ester of a diacetone hexuronic acid.

$C_{13}H_{20}O_7$.	Calculated.	C 54.13,	H 7.0,	OCH_3 10.76
	Found.	" 54.29,	" 7.11,	" 10.79

Catalytic Reduction of Methyl Ester of Diacetone d-Galacturonic Acid—5 gm. of copper chromite catalyst were added to a solution of 5 gm. of the methyl ester of diacetone *d*-galacturonic acid in 100 cc. of absolute methanol. This mixture was placed in a high

[2] Niemann, C., and Link, K. P., *J. Biol. Chem.*, **104**, 195 (1934).

pressure reduction apparatus and a hydrogen pressure of 3000 *pounds per sq. inch was applied.* The temperature was now *slowly increased to 175°. At this temperature the pressure rose* to 4300 pounds per sq. inch. After the reaction had proceeded during 5 hours at this temperature, the whole apparatus was allowed to cool for 18 hours.

The reaction mixture was removed and the catalyst separated by filtration. The filtrate was treated with charcoal and again filtered in order to remove the last traces of catalyst. A colorless sirup was obtained upon concentration of this filtrate. Yield 4.5 gm.

The sirup was now distilled at a bath temperature of 120° and a pressure of 0.17 mm. Only one fraction was collected. Yield 4.3 gm. This material was then redistilled and four fractions were collected at a bath temperature of 115° and a pressure of 0.13 mm. The first fraction was taken for analytical data. It had a refractive index of $n_D^{25} = 1.4655$ and a specific rotation of

$$[\alpha]_D^{25} = \frac{-5.04° \times 100}{2 \times 4.240} = -59.4° \text{ (in chloroform)}$$

The substance had a composition agreeing approximately with that calculated for a diacetone hexose.

4.227 mg. substance: 8.595 mg. CO_2 and 2.975 mg. H_2O

$C_{12}H_{20}O_6$. Calculated, C 55.35, H 7.7; found, C 55.44, H 7.9

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THE REDUCTION OF AMINOSORBITOL HYDROCHLORIDE WITH HYDRIODIC ACID

BY P. A. LEVENE AND CLARENCE C. CHRISTMAN

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(Received for publication, December 6, 1937)

Inasmuch as attempts at the reduction of glucosaminic acid to optically active norleucine have met with great difficulties, it was decided to select a different approach to the solution of the problem of the configuration of glucosamine; namely, the conversion of glucosamine into 2-aminopentahydroxyhexane[1] (2-aminosorbitol) in the hope that by reduction with hydriodic acid the hydroxyamine would be converted into 2-aminohexane. Thus a direct answer to the configuration of *d*-glucosamine would be furnished, since the configuration of 2-aminohexane has been correlated by Levene and Mardashew[2] to that of 2-aminocaproic acid. The work has not yet been completed but the results so far obtained are reported here in view of the activities of other laboratories on the question of the configuration of *d*-glucosamine.

The product so far obtained by reduction with aqueous hydriodic acid has the composition of 2-aminohexene oxide; on acetylation it forms a monoacetyl derivative which no longer possesses a free amino group but on deacetylation the amino group becomes free again. This acetylated substance possesses no ethylenic linkage and is not a double molecule formed by union of 2 monohydroxyaminohexane molecules, since a molecular weight of 166 was obtained by the Rast method. The composition of the substance, therefore, is $C_6H_{13}ON \cdot HCl$.

Further reduction of this substance is now in progress. Also, other methods of arriving at the configuration of the 2-aminopentahydroxyhexane are now in progress.

[1] Levene, P. A., and Christman, C. C., *J. Biol. Chem.*, **120**, 575 (1937).
[2] Levene, P. A., and Mardashew, S., *J. Biol. Chem.*, **117**, 707 (1937).

It may be mentioned that reduction of the hexaacetate of 2-aminopentahydroxyhexane with hydriodic acid in glacial acetic acid resulted in a 2-aminomonohydroxyhexane directly and its acetate by acetylation of the substance from the mother liquor. Unfortunately the yield was so small that the experiment could not be repeated with sufficiently consistent success to warrant further experimentation.

EXPERIMENTAL

Treatment of Aminosorbitol Hydrochloride with Hydriodic Acid—A solution of 5 gm. of aminosorbitol hydrochloride[1] in 60 cc. of hydriodic acid (sp. gr. = 1.70) was heated in a sealed tube during 5 hours at a temperature of 125°. The furnace and tube were allowed to cool for 18 hours, after which the tube was opened and the contents diluted with water to 180 cc.

The solution, containing much free iodine, was evaporated under diminished pressure to about 75 cc. and then diluted to 1000 cc. with water. The major portion of the free iodine and hydriodic acid was removed by adding lead carbonate and then filtering off the lead salts. (In this and in all the following operations the precipitates were shaken with water and then filtered, the filtrates being combined with the original filtrate.) The remainder of the iodine and hydriodic acid was removed with silver carbonate in the presence of free sulfuric acid. After removal of all the silver and lead ions with hydrogen sulfide, the solution was made alkaline and steam-distilled into dilute hydrochloric acid. The distillation was continued until the distillates were no longer alkaline to red litmus paper. The acid solution of the distillate was evaporated under diminished pressure to a sirup, which was dried by repeated addition and concentration of benzene and absolute ethyl alcohol. The dried sirup was dissolved in absolute ethanol, filtered from the ammonium chloride, and an equal volume of ether added to the filtrate. More ammonium chloride separated out after standing in the refrigerator overnight and this was also filtered off. The sirup obtained by concentration of this filtrate was used in the next experiment.

Preparation of Crystalline Chloroplatinate from Reaction Product Obtained by Treatment of Aminosorbitol Hydrochloride with Hydriodic Acid—The dried sirup from the previous experiment was

dissolved in a small volume of absolute ethanol and an excess of chloroplatinic acid added. Ammonium chlóroplatinate, if present, was removed by filtration and the filtrate concentrated to half its volume in a vacuum desiccator.

In this way a crop of crystals was obtained which were recrystallized by dissolving in warm absolute ethanol, filtering, and then allowing the filtrate to evaporate spontaneously in a desiccator. The average yield obtained in several experiments was about 1.5 gm. (from 5 gm. of aminosorbitol hydrochloride).

This chloroplatinate had a composition which agreed fairly well with that calculated for the chloroplatinate of an aminohexene oxide. However, the analytical data varied with different preparations (indicating the presence of other material). By repeated careful recrystallization a product was obtained which had the following composition.

4.780 mg. substance: 3.902 mg. CO_2 and 1.895 mg. H_2O

$C_{12}H_{28}O_8N_2PtCl_6$.	Calculated.	C 22.48, H 4.5
	Found.	" 22.26, " 4.4

Preparation of 2-Aminohexene Oxide Hydrochloride—9 gm. of the once recrystallized chloroplatinate (from the previous experiment) were dissolved in 100 cc. of warm water. Hydrogen sulfide was passed into the solution for 3 hours and the mixture allowed to stand overnight in the presence of hydrogen sulfide. The platinum sulfide was removed by filtration and well washed with warm water. The combined filtrates were concentrated to 25 cc., treated with charcoal, and then filtered.

This filtrate was concentrated to a dry crystalline mass which was dried further by frequent addition and evaporation of benzene and absolute alcohol. The product was obtained in the pure state by recrystallizing from a small volume of absolute ethanol. Yield 2.0 gm. All the mother liquors were concentrated to dryness. Yield 1.4 gm.

The pure substance had a melting point of 217–218° and a specific rotation of $[\alpha]_D^{25} = \frac{-0.30° \times 100}{2 \times 2.54} = -5.9°$ (in absolute ethanol). It is soluble in alcohol and water but practically insoluble in ether, acetone, chloroform, and pentane.

The compound had a composition agreeing with that of 2-aminohexene oxide hydrochloride.

3.981 mg. substance: 6.914 mg. CO_2 and 3.330 mg. H_2O
6.078 " " : 0.498 cc. N_2 (748 mm. at 28°)
7.478 " " : 4.90 cc. 0.01 N $AgNO_3$

$C_6H_{13}ON \cdot HCl$.	Calculated.	C 47.49,	H 9.3,	N 9.20,	Cl 23.08
	Found.	" 47.36,	" 9.36,	" 9.14,	" 23.26

Attempted Catalytic Hydrogenation of 2-Aminohexene Oxide Hydrochloride—200 mg. of crystalline 2-aminohexene oxide hydrochloride dissolved in 30 cc. of absolute ethanol were shaken with Adams' catalyst and hydrogen for several hours. The catalyst was removed by filtration and the filtrate concentrated to about 4 cc. Unchanged starting material was isolated from this solution by the addition of ether, as indicated by the melting point (217–218°) and analysis, thus indicating the presence of an oxygen ring and the absence of any double bonds.

Acetylation of 2-Aminohexene Oxide Hydrochloride—Pure 2-aminohexene oxide hydrochloride (0.6 gm.) was refluxed for 2 hours with 10 cc. of acetic anhydride and 1 gm. of freshly fused sodium acetate. The mixture was allowed to stand overnight at room temperature and then concentrated to dryness under reduced pressure. All traces of acetic anhydride and acetic acid were removed by repeated concentrations with the addition of small volumes of benzene.

The crystalline mass was now treated with 15 cc. of chloroform and filtered. The filtrate was dried with sodium sulfate and concentrated to a dry mass of crystals. The product was recrystallized by dissolving in an acetone-ether mixture and then adding a small volume of pentane. Yield 0.55 gm. The substance was pure after three such recrystallizations and had a melting point of 142–143°. It had the following specific rotation.

$$[\alpha]_{\mathrm{D}}^{[illegible]} = \frac{+0.29^\circ \times 100}{2 \times 3.56} = +4.1^\circ \quad \text{(in absolute ethanol)}$$

The substance showed no perceptible rotation in chloroform solution. It is soluble in acetone, ether, chloroform, water, and benzene but is practically insoluble in pentane.

The material had a composition agreeing with that of an N-acetylaminohexene oxide. It contained no free amino nitrogen and a Rast molecular weight determination gave a value of 166, which is in accord with the calculated value of 157.

4.594 mg. substance: 10.310 mg. CO_2 and 3.897 mg. H_2O
5.994 " " : 0.455 cc. N_2 (759 mm. at 25°)
5.402 " " : 3.371 " 0.01 N $Na_2S_2O_3$*

$C_8H_{15}O_3N$.	Calculated.	C 61.14, H 9.6, N 8.9, $COCH_3$ 27.39
	Found.	" 61.20, " 9.5, " 8.7, " 26.83*

Action of Hydrogen Iodide in Glacial Acetic Acid on Aminosorbitol Hydrochloride—Two sealed tubes, each containing 5 gm. of aminosorbitol hydrochloride, 0.5 gm. of phosphonium iodide, and 40 cc. of a solution composed of equal parts by weight of dry hydrogen iodide and dry glacial acetic acid, were heated at 125° for 5 hours. The materials were united and the product isolated in the same way as in the previous experiment with hydriodic acid.

The dried, steam-distilled sirup was treated with chloroplatinic acid but only about 0.2 gm. of crystalline chloroplatinate was obtained.

Action of Hydrogen Iodide in Glacial Acetic Acid on 2-Aminosorbitol Hexaacetate[1]—To 6 gm. of pure 2-aminosorbitol hexaacetate were added 25 cc. of a solution composed of equal parts by weight of dry hydrogen iodide and glacial acetic acid, and the mixture sealed in a bomb tube. After heating at 125° during 4 hours and then cooling in the furnace for 12 hours, the tube was opened and the contents diluted with 150 cc. of water.

Sulfur dioxide was passed into the solution until all the free iodine had been consumed. The excess sulfur dioxide was removed by aspirating with air and the sulfuric acid was removed quantitatively by the addition of barium hydroxide solution. This solution was then concentrated to dryness under diminished pressure at 40°, and the residue was dissolved in 100 cc. of methyl alcohol and reduced with hydrogen and Raney's catalyst.

When the reduction was complete, the catalyst was removed by filtration and the methyl alcohol solution steam-distilled in alkaline solution until the final distillate was no longer alkaline to red litmus. The distillate was collected in a solution of hydrochloric acid in order to prevent the loss of the volatile free base.

* This substance has the acetyl group bound so firmly to the nitrogen that the time of digestion in the acetyl determination had to be increased (from 3) to 6 hours. This is the first compound which Dr. Elek has found to require more than 3 hours for completion of the deacetylation.

*The acid distillates were then concentrated to dryness under re-*duced pressure at 40° and a small amount of crystalline material was obtained by dissolving the partially crystalline residue in acetone and then adding ether. Yield 0.05 gm. This was recrystallized twice from acetone and then melted at 86–88°.

The substance had the following composition.

4.299 mg. substance: 7.302 mg. CO_2 and 3.995 mg. H_2O; 2% ash
5.120 " " : 0.394 cc. N_2 (760 mm. at 27°)
$C_6H^{15}ON \cdot HCl$. Calculated. C 46.9, H 10.45, N 9.12
Found (ash-free). " 47.1, " 10.5, " 8.92

The acetone-ether mother liquors were combined and evaporated to dryness. The dry sirup was refluxed for 1 hour with a mixture of anhydrous sodium acetate and acetic anhydride, and then kept at room temperature for 1 day. This mixture was now concentrated to dryness and the product extracted with chloroform. The chloroform extract was washed with three small portions of water and then dried with anhydrous sodium sulfate.

The product partly crystallized after the removal of the chloroform and was completely crystallized from ether and pentane. Yield 0.25 gm. After one more recrystallization from ether and pentane the substance melted at 77–78° and had the following specific rotation.

$$[\alpha]_D = \frac{+1.62° \times 100}{1 \times 4.08} = +39.7° \quad \text{(in chloroform)}$$

This material had the following composition.

4.416 mg. substance: 9.590 mg. CO_2 and 3.790 mg. H_2O
6.420 " " : 6.36 cc. 0.01 N $Na_2S_2O_3$
$C_{10}H_{19}O_3N$. Calculated. C 59.9, H 9.5, $COCH_3$ 42.8
Found. " 59.2, " 9.6, " 42.6

[Reprinted from The Journal of Biological Chemistry, March, 1938,
Vol. 123, No. 1, pp. 83–85]

THE REDUCTION OF GLUCOSAMINIC ACID WITH HYDROGEN IODIDE IN GLACIAL ACETIC ACID

By P. A. LEVENE and CLARENCE C. CHRISTMAN

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(Received for publication, December 6, 1937)

Although the configuration of glucosamine formulated by one of the present authors (P.A.L.) has been substantiated in the last few years by Bergmann *et al.*[1] and more recently by Karrer and Meyer,[2] yet all the evidence thus far furnished has been based on indirect evidence and, in addition, is contradicted by the conclusion of Neuberg, Wolff, and Niemann[3] reached by the method of classical organic chemistry. However, the experiments of Neuberg could never be duplicated. Levene and Wildman[4] treated every one of the eight *d*-2-aminohexonic acids according to the directions of Neuberg, Wolff, and Niemann[3] but the 2-aminohydroxy acid obtained by them in the first phase of Neuberg's process was invariably inactive. The attempts of Karrer and Meyer[5] to repeat Neuberg's experiments likewise met with little success.

It seemed that if the formation of the 2-aminohydroxycaproic acid could be accomplished without complete racemization, then its transformation into optically active norleucine would be promising. Optically active 2-aminohydroxycaproic acid has now been obtained from glucosaminic acid by reduction with hydriodic acid dissolved in glacial acetic acid. Great difficulties, however, were encountered in the preparation of pure derivatives

[1] Bergmann, M., Zervas, L., Rinke, H., and Schleich, H., *Z. physiol. Chem.*, **224**, 33 (1934).

[2] Karrer, P., and Meyer, J., *Helv. chim. acta*, **20**, 407 (1937).

[3] Neuberg, C., Wolff, H., and Niemann, W., *Ber. chem. Ges.*, **35**, 4009 (1902).

[4] Levene, P. A., and Wildman, E. A., unpublished work.

[5] Karrer, P., and Meyer, J., *Helv. chim. acta*, **18**, 782 (1935).

of this hydroxy acid, owing to simultaneous formation of a lactone, whose ready formation may be taken as an indication that the hydroxyl group in the hydroxy acid is situated on carbon atom (4). Thus the substance probably has the following structure, $CH_3 \cdot CH_2 \cdot CH(OH) \cdot CH_2 \cdot CH(NH_2) \cdot COOH$.

Efforts to reduce this substance to optically active norleucine are being continued.

EXPERIMENTAL

Action of Hydrogen Iodide in Acetic Acid on Glucosaminic Acid—Four sealed bomb tubes, each containing 5 gm. of glucosaminic acid (finely powdered), 0.5 gm. of phosphonium iodide, and 30 cc. of a solution composed of equal parts by weight of dry hydrogen iodide and dry glacial acetic acid, were heated at 125° during 4 hours. The tubes were then opened, and the contents removed, combined, and diluted to 1 liter with water. The aqueous solution was now extracted several times with chloroform in order to remove the excess iodine.

In the following operations all the lead and silver precipitates and residues were triturated and washed several times with hot water. These washings were then added to the filtrate from which the precipitate or residue had been removed. All concentrations were performed at 40–50° under reduced pressure.

The aqueous solution, after chloroform extraction, was treated with washed lead carbonate until nearly free from halogen. The lead precipitate was removed by filtration and the filtrate and washings treated with silver carbonate until free of halogen. The silver residues were then removed by filtration and the silver and lead ions removed from this filtrate by means of hydrogen sulfide.

The solution was now concentrated to 200 cc. and again treated with silver carbonate and hydrogen sulfide. The filtrate was concentrated to a thick sirup which crystallized upon the addition of 200 cc. of absolute ethyl alcohol. The material was removed by filtration, and the filtrate diluted with water and again treated with silver carbonate and hydrogen sulfide. Another crop of crystals was obtained by adding alcohol again to the concentrated filtrate. Total yield 5.6 gm.

The product was recrystallized by dissolving in the minimum amount of cold water and then adding an excess of absolute

ethyl alcohol. In this way 4.9 gm. of material were obtained which had the following rotation.

$$[\alpha]_D^{25} = \frac{-1.70° \times 100}{2 \times 5.186} = -16.4° \text{ (in 20\% hydrochloric acid)}$$

The substance had a composition agreeing with that for monohydroxyaminocaproic acid.

4.892 mg. substance: 8.718 mg. CO_2 and 3.789 mg. H_2O; 0.6% ash
23.30 " " : 4.16 cc. N_2 (762 mm. at 25°, Van Slyke)

$C_6H_{13}O_3N$.	Calculated.	C 49.00, H 8.9, amino N 9.52
	Found (ash-free).	" 48.88, " 8.7, " " 9.92

[Reprinted from COMPTES-RENDUS DES TRAVAUX DU LABORATOIRE CARLSBERG, 1938, Vol. 22, série chimique, pp. 347–356. JUBILEE VOLUME IN HONOR OF PROFESSOR S. P. L. SØRENSEN]

THE PENTACYANO-AQUO COMPLEXES OF IRON

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(Received for publication, July 21, 1937)

It was first observed by Davidsohn (1) that the oxidation-reduction potential of the system pentacyano-aquo-ferriate and -ferroate does not follow the law holding for similar reversible ferri-ferro systems. The curve of the potential plotted against the degree of oxidation is very much steeper than in other cases. His interpretation was the assumption of a compound on a level of oxidation intermediate between the ferri and the ferro compound, which he suggested was a compound of two molecules of the ferri with the three molecules of the ferro form. Michaelis and Smythe (2) confirmed the general feature of Davidsohn's observation but arrived at the conclusion that these complexes always exist as double molecules, in three levels of oxidation: a ferri-ferri compound, a ferri-ferro compound, and a ferro-ferro compound. The system was accordingly interpreted by the latter authors to be a two-step oxidation-reduction system comparable to those of organic dyestuffs in which a semiquinone is the intermediate compound. The analogy consists in the fact that all three levels of oxidation are represented by molecules of the same molecular size. The intermediate compound is, however, not a radical in this case.

In the meantime many two-step systems have been systematically investigated both theoretically and experimentally. Utilizing the experience gained by these studies the authors found some features of their previous results not quite compatible with the theory and decided to study this system over again. In fact, the titration curves now obtained and some other methods used will lead to a modification of the previous interpretation. We may attribute the slight but characteristic alterations of the results to improvements in purification of the material.

Preparation of material. Both the sodium pentacyano-aquo-ferroate and ferriate were prepared according to Hofman (3). We

were able to obtain both compounds in crystalline form. The ferriate forms long, reddish-blue needles and the ferroate very small yellow needles—so small that they are kept in brownian movement. Of the two compounds the ferriate is much easier to purify and crystallize. It is soluble in absolute methyl alcohol and in this way can be separated from the impurities present. No such fortunate property is known for the ferroate. Our crude preparations of ferroate always contained a small amount of some blue substance—possibly a breakdown product—that was quite difficult to separate. In addition, the ferroate is autoxidizable so it must be protected from oxygen. By repeatedly dissolving in water and precipitating with alcohol in the absence of oxygen, the compound, which originally separates as an oil, can be obtained in pure form and induced to crystallize.

Both compounds are hygroscopic. The ferriate when precipitated from an absolute methyl alcohol solution by the addition of ether and placed for a short time over H_2SO_4 in vacuo contains 27.6 % N. The theoretical (sodium salt) is 28.0 %. The ferroate, when precipitated from aqueous solution by alcohol, requires much longer drying.

Potentiometric titration. The potentiometric method consists in a titration of the ferri compound by a suitable reductant, either a leuco-dye, such as that of Rosindulin GG, which gives a sharp titration endpoint due to its very negative potential range, or ascorbic acid, which though not giving quite as sharp an endpoint is still satisfactory and, being itself colorless, allows of the observation of color changes. Oxidative titrations of the ferro compounds are much less satisfactory. Bromine or chlorine water are oxidants of sufficiently positive potential range, but they not only oxidize the ferro compound to the ferri compound but in addition cause, to a smaller or greater extent according to conditions, irreversible side reactions. This can be seen from the fact that when the ferro compound, dissolved in the solvent used for the titration experiments is titrated with Br_2 or Cl_2 the color at the endpoint is not the same red-violet as in a solution of the pure ferri compound but more or less shifted to blue. The titration curves obtained with Br_2 or Cl_2 are not reproducible in all details, although the general steepness of the whole curve is the same as in reductive titration. The latter are very much better reproducible and will be discussed in detail.

In reductive titration, the red-violet color of the ferri compound turns to an indefinable grey as early as at 15–20 % of the total reduction, to grass-green at about 30 % and fades gradually from here on to the pale yellow of the ferro form, which is reached at the end of the titration. On reoxidation by Br_2 the violet color returns, with the restriction that it may be somewhat shifted to blue violet, in accordance with the above discussion. The color change during reduction shows that the substance exists in more than two oxidation-reduction

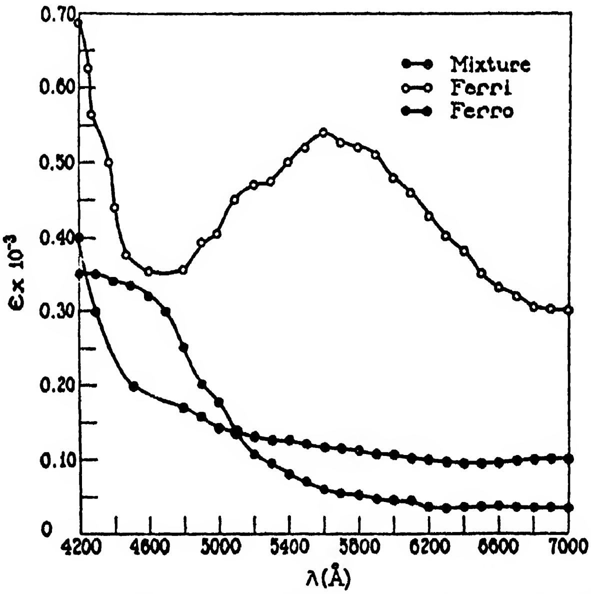


Fig. 1. Extinction coefficient of sodium pentacyano-aquo-ferriate, the corresponding ferroate, and of an equimolecular mixture of the two, according to measurements by Dr. G. I. Lavin, of the spectroscopic laboratory of The Rockefeller Institute.

levels. There are at least three levels, but possibly more. This is confirmed by a spectroscopic analysis. The characteristic, though rather diffuse band of the ferri compound in the visible range with a maximum at 5600 Å, is entirely lost when the ferri compound is mixed with an equal amount of the ferro compound. This would not be possible if on mixing the two compounds, both remained in the solution as a simple mixture without interaction.

In reductive titrations the potentials are established quite satisfactorily, although some comments are necessary. This complex is,

quite generally, less stable than the hexacyano complex. An aqueous solution of the ferri compound gradually fades out on standing; rather quickly in an alkaline solution, and after several days in acid solution (p_H 4.6). In the latter case an insoluble blue-green sediment is formed after 3 or 4 days. Such a substance cannot be expected to give strictly constant potentials. On working at p_H 3 to 6, the drift, after the establishment proper of the potential, does not amount to more than $\frac{2}{10}$ to $\frac{5}{10}$ of a millivolt per minute. Since the whole titration curve is very steep, no appreciable error can be caused by such a slight drift. The drift is always toward the negative side in the first half of the titration and usually toward the positive side in the second half. The establishment of the potential to this state of approximate constancy is not really instantaneous. It is a good compromise to take as potentials the readings 5 minutes after each addition of the reductant. If we chose 2, or 10 minutes instead, the difference of the results is so small that it could not be noticed in a graph of the scale used in figure 2. In this way it was ascertained by a great many experiments that the titration curve is much steeper than that of a bivalent oxidation-reduction system of normal behavior. In addition the middle portion is much steeper than the rest.

This jump in the middle portion of the curve, in a few experiments, was precisely at 50% of the reduction. In most cases it was slightly displaced so that the point of inflection seemed to be located at about 45% to 47%. This slight flaw of symmetry in the curve is very probably an artefact caused by the coincidence of at least two factors acting in the same direction. Firstly, there is no guarantee that the ferri compound, even if crystalline, has been freed, by the recrystallization, from all traces either of the unknown by-product mentioned above, or of a compound of a somewhat lower state of oxidation, which will be presently discussed. The latter assumption is supported by the fact that the steepness of the titration curve in the very beginning of the titration is often smaller than it should be. One has the impression that the true zero point of the curve should be extrapolated somewhat to the left hand side of the experimental zero point. In the second place, at the very end of the titration the establishment of the potential, as it just begins to overlap with the potential range of the reducing agent, is sluggish, the drift is slow and directed toward

the negative side, and the readings of the potentials may be a little more positive than those corresponding to equilibrium conditions. These two factors would tend to cause a shortening of the first half, and a stretching out of the second half of the titration curve. These factors justify, we believe, placing the real point of inflection at 50% and taking the very slight deviations that may occur as artefacts. It is quite possible that the much stronger asymmetry of the titration curve as observed by Davidsohn is due to the same causes.

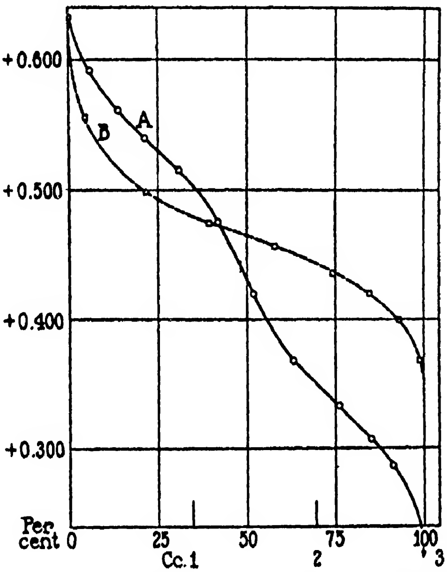


FIG. 2. Curve A: 5 mg. of sodium pentacyano-aquo-ferriate are dissolved in 25 cc. of acetate buffer, $\frac{1}{10}$ molar in Na acetate, and having a pH of 4.6. The solution is made $\frac{1}{10}$ molar in KCl by addition of the solid salt and then titrated with leuko-rosindulin GG at 30°C. Ordinate: potential, referred to normal hydrogen electrode, in millivolts. Abscissa: cc. of added reductant, and percentage of reduction.

Curve B: A comparative experiment with potassium ferricyanide under the same conditions. Abscissa: per cent. of reduction.

As we have to deal with polyvalent ions the valence of which will change on oxidation-reduction, it is important to maintain the ionic strength during the titration by adding an excess of KCl. In general, we have two sets of experiments; one in M/10 KCl, the other with M/1 KCl, and the concentration of the complex was varied from 0.0003 to 0.001 molar, the molecular weight being counted as the one of a non-polymerized molecule. The shape of the whole curve is the same

with $\frac{1}{10}$ M and $\frac{1}{1}$ M KCl; the potential at 50% reduction, however, is about 50 millivolts more positive in M/1 than in M/10. This is, approximately the same difference as in the system K_3 ferricyanide/K_4 ferrocyanide (4).

The dependence of the potential on p_H as found in our previous paper is not confirmed. With our present, purer preparations, the potentials were the same on varying the p_H from about 3 to 7. Higher p_H's were avoided because of the lability of the compound. The observation that the potentials are the same from p_H 3 to 7 at least, is in agreement with the fact that on acidimetric titration with the glass electrode, of either the disodium-ferri compound, or the trisodium-ferro compound, no acidic ionization constant could be detected of the magnitudes $\gtrless 10^{-3}$. It is true that there is an acidic constant of the ferro compound, for the yellow aqueous solution turns blue on the addition of a strong acid. This is a reversible change but the p_K must be < 3; perhaps even smaller than 2. It never manifests itself in our titration experiments.

Interpretation of the titration curve. The essentially striking feature of the titration curve is its steepness which may be contrasted in Figure 2 with a curve of a normal one-electron system, ferricyanide-ferrocyanide. In the middle section the slope is especially steep. Obviously at 50% of the reduction a step between two levels of reduction exists, with very little overlapping. This being so, we may consider the half from 0–50%, and the other half from 50–100%, separately. The one half is a repetition of the other, the first being on a higher level of potential than the second.

Each half is symmetric around its own midpoint (which lies at 25%, resp. 75% of the total reduction). The index potential of each half (i.e., the potential difference between 25% and 12.5%, or between 37.5% and 25%; and between 75% and 63.5%; or between 87.5% and 75% of reduction) is 36 millivolts at 30°C. The probable error of this figure may be estimated at ± 2 or 3 millivolts. This is incompatible with our previous assumption that there are in all only three levels of oxidation. In this case, the first and second levels should differ by one electron; and so should the second and the third. But in this case the index potential for each half should be 28.6 millivolts. Since the index potential is 36, each of the halves must be at least a

two-step system, in which, however, the two steps distinctly overlap. The symmetry of the whole titration curve around its midpoint, and the symmetry of each half around its own midpoint, indicates that the size of the molecule is not changed during the course of reduction. This assumption is confirmed by the fact that the shape of the whole curve is independent of the initial concentration of the compound, on varying the concentration four times and more. We must then assume that the compound has four times its lowest possible molecular weight, and thus five compounds at different levels of oxidation can exist:

I. 4 (Na_2X)
II. 3 (Na_2X), 1 (Na_3X)
III. 2 (Na_2X), 2 (Na_3X)
IV. 1 (Na_2X), 3 (Na_3X)
V. 4 (Na_3X)

X = $Fe(CN)_5H_2O$.

In such a system we can distinguish several partial normal potentials. What we may call the mean normal potential of the system is the potential at 50% reduction, where (II) = (IV), and at the same time (I) = (V). (Brackets designate concentrations.) The distinct jump in this midpoint of the curve indicates that (II) and (IV) must be very small at this point; and that (I) and (V) are even vanishingly small. Furthermore, at 25% reduction (I) = (III); this potential may be called the normal potential of the partial system (I)/(III). Likewise, at 75% reduction the potential is the normal potential of the partial system (III)/(V). Furthermore, the partial normal potentials of the systems (I)/(II), (II)/(III), (III)/(IV), and (IV)/(V), may be distinguished, and, if desired, properly located according to the general rules presented previously. During the titration, between 0 and 50%, (I), (II), and (III) always coexist. At precisely 50%, (II) and (IV) are equal but very low; (I) and (V) are equal but vanishingly low, and only (III) exists practically. Between 50 and 100%, (III), (IV), and (V) coexist.

The fact that the titration curve is especially steep around its midpoint is equivalent to saying that the dismutation constant of the most intermediate form is smaller than that of the others. This means that (III) is a more stable compound than (II) or (IV). This

is quite plausible because of the greater symmetry of (III) which contains just the half of the iron in the ferro state and the other half in the ferri state.

Additional Evidence for the Quadrimolecular Size

It seemed desirable to have supplementary evidence for the quadrimolecular form. The most direct evidence would be a molecular weight determination and we have attempted to get this by the diffusion method of Anson and Northrop (5). A solution 0.02 m. with K_3FeCN_6 and 1.0 m. with KCl was used as reference and a solution of our ferri compound as the sodium salt, 0.02 m. with respect to iron and 1.0 m. with NaCl was compared with it. The solutions were allowed to diffuse into 20 cc. of the appropriate 1.0 m. salt solution for periods of 2.5 hours at 30°C. The excess of salt is to eliminate liquid junction potentials and to abolish those electromotive forces which would change the law of diffusion for an ion as compared with that of a non-electrolyte. The amount diffused was determined colorimetrically with a stufen photometer under conditions where it was shown that both compounds obeyed Beer's law. The amount diffused is expressed in terms of cc. of the diffusing solution (5). The results for two successive periods in each of two experiments (all with the same membrane) are given in table I.

Substance	Experiment	Period	Qcc.	Average Qcc.
K_3FeCN_6	1	1	0.203	
—	1	2	0.230	
—	2	1	0.251	
—	2	2	0.246	
				0.233
$Na_2FeCN_5H_2O$	1	1	0.115	
—	1	2	0.131	
—	2	1	0.108	
—	2	2	0.111	
				0.117

Ferricyanide ion has a molecular weight of 211.8. On the assumption that the rate of diffusion is inversely proportional to the square root of the molecular weight, the above data give a value of 837 for the molecular weight of the pentacyano-aquo ion. The calculated value

is 203.8 for the monomolecular form. The result thus clearly indicates a quadrimolecular form.

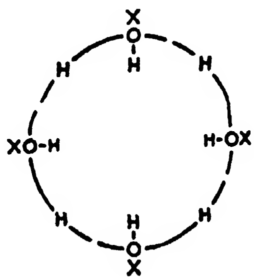
One other method has been used. The threshold value of the concentration at which an anion just coagulates a positively charged lyophobic colloid, such as colloidal Fe_2O_3, depends largely on the number of charges on the ion. On comparing ions of similar chemical nature, but differing in the number of charges one expects the threshold value to decrease as the number of charges increases. Using a solution containing 0.5% colloidal Fe_2O_3 we find the threshold value for K_3FeCN_6 to be 0.00080 m. This and all following figures are reproducible approximately to ± 12%. Under the same conditions we find a value of 0.00018 m. for K_4FeCN_6. We may now compare $Na_2FeCN_5H_2O$ with these. If it is monomolecular, it is bivalent and should have a threshold higher than that for K_3FeCN_6. Actually we find the value to be much lower, namely 0.000313 m. with respect to iron. If we consider this compound to be bimolecular it is now quadrivalent and the threshold value is 0.000156 m. Thus it appears to be at least equal to and probably even a little stronger in coagulating power than the K_4FeCN_6 with which it should be comparable. If we consider it to be quadrimolecular it is octavalent and the threshold value is 0.000078 m. This shows it to be a stronger coagulant than ferrocyanide. We may conclude that this method shows the pentacyano-aquo complex to be at least bimolecular, but possibly in even a higher state of association.

Thus of the three methods used, two definitely indicate a quadrimolecular formula and the third definitely rules out a unimolecular size and is at least compatible with a quadrimolecular one. We must mention, however, that neither of these two latter methods definitely excludes the possibility of the preparation being a mixture of iron-containing compounds in such proportions that the average number of iron atoms is four. However, to explain how such a mixture could give an oxidation-reduction curve like the one reported, one must make so many assumptions that we greatly prefer to consider it a quadrimolecular compound.

Tentative Explanation of the Results

An attempt to explain the quadrimolecular arrangement is this. One of the six atom groups coordinatively bound to the iron is H_2O.

In ordinary liquid water the molecules are supposed to be linked by hydrogen bonds. We introduce an auxiliary hypothesis, made ad hoc indeed, namely, that the hydrogen bond of a coordinatively bound H_2O molecule such as this one attached to iron, is stronger with another coordinatively bound H_2O than with free H_2O. Then, two molecules of the compound containing the coordinatively bound water will combine. In the same way this double molecule combines with another single molecule, and so on until the flexibility of the chain allows the closing of the ring. In this case the ring would be an eight-membered one as shown:



X is either $Fe(CN)_5$ or $Fe(CN)_5$.

Whether such an interpretation will stand the test can only be shown by further theoretical and experimental evidence from other sources.

SUMMARY

A potentiometric analysis of the reductive titration curve of sodium pentacyano-aquo-ferriate to sodium pentacyano-aquo-ferroate can be best interpreted by assuming that each of these two complexes is in solution present as a quadrimolecular aggregate. Between the ferri and the ferro form there are three intermediate forms in which a part of the four Fe atoms is in the ferri and a part in the ferro state. The evidence is strengthened by observation of color change, rate of diffusion, and the threshold value of coagulation of Fe_2O_3-sol. An attempt is made to explain the molecular aggregation on the basis of hydrogen bonds between the coordinatively bound water molecules leading to an eight-membered ring.

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A NOTE ON THE STAINING OF TUBERCLE BACILLI IN SECTIONS

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It is commonly believed that fixation of tissues in formalin renders them unsuitable for staining acid-fast bacteria. A method for staining tubercle bacilli which gives satisfactory results after formalin and after Zenker-formol seems, therefore, worth recording. The method which follows yields good staining of the bacteria with either of these fixatives. With formalin-fixed tissue the green counterstain is prone to fade in a few months, the bacteria and nuclei remaining well stained indefinitely. With tissue fixed in Zenker-formol, all structures retain the dyes for prolonged periods.

In general the method consists of staining in iron hematoxylin, followed by fuchsin, and employing light green as a counterstain. This gives a clear staining of the nuclei; the nuclear membrane is dark blue green and the nucleoli are blue green, with a paler light green for the cytoplasm against which the red bacilli show up brilliantly.

PROCEDURE

1. Run slides down to water as usual and rinse in distilled water.
2. Mordant in iron alum solution in the oven for five minutes (45° to 50°C.).
3. Rinse at the tap.
4. Stain in hematoxylin, also in the oven, for five minutes.
5. Put directly into the picric acid for five minutes, or longer if necessary, until the stain remains only in the nuclei.
6. Wash thoroughly in running water (fifteen minutes or longer) until the sections are thoroughly free of the picric acid.
7. Place on the heating bar and pour the fuchsin solution over

them. Heat until steam comes off, but do not boil. Let cool for three minutes, heat again, and again allow to cool for three minutes.

8. Destain in acid alcohol for a few seconds, until the fuchsin starts coming off in clouds; rinse in tap water and continue destaining until the sections are barely pink.

9. Rinse in water and put into alkaline water for a few seconds.

10. Wash thoroughly in running water for ten minutes or more.

11. Stain in the light green solution for five minutes.

12. Rinse in water and run up quickly through the alcohols and xylol; mount as usual.

1. *Iron alum.*—5 per cent aqueous solution of iron alum.

2. *Hematoxylin.*—Dissolve 1 gm. of Grübler's hematoxylin in 80 c.c. of hot distilled water, cool, and add 10 c.c. of glycerin and 10 c.c. of 95 per cent alcohol.

3. *Picric acid.*—Add 2 parts of a saturated alcoholic (95 per cent) solution of picric acid to 1 part of 95 per cent alcohol.

4. *Fuchsin.*—Add 16 c.c. of a saturated alcoholic (95 per cent) solution of fuchsin to 84 c.c. of aniline water.

5. *Acid alcohol.*—3 per cent nitric acid in 95 per cent alcohol.

6. *Alkaline water.*—Add a little ammonia to distilled water.

7. *Light green.*—1 per cent aqueous solution of light green (Grübler).

DISCUSSION

This method has been used over a period of three years and has been applied to tuberculous tissues from several animal species inoculated with various strains of tubercle bacilli. Aside from technical facility, it has certain other distinct advantages. It gives good nuclear and cytoplasmic staining, so that fine cellular differentiation is obtained. This is particularly evident in such tissues as bone marrow, in which sections so stained are useful not only for studies of tubercle bacilli but also for studies of the marrow cells.

The method is excellent for purposes of microphotography, as tubercle bacilli are stained brilliantly red while nuclei and cytoplasm take different values of green so that excellent contrast is obtainable. For ordinary microscopic examinations, the red-green contrast has been found superior to other staining methods.

By this method tubercle bacilli are equally well stained in sections

from all viscera. Whereas certain other methods have been found unsatisfactory for staining these organisms in brain and meninges, the technique described herein has been found highly satisfactory in a large series of animals subjected to intracerebral inoculation. Intracellular organisms, so often appearing purple or black when stained by other methods, are brilliant red in this method and are, therefore, easily distinguishable from nonacid-fast bacteria or nonbacillary material. There has been no evidence that bacteria other than tubercle bacilli retain the fuchsin; moreover, distinctive characteristics of individual bacteria, such as beading, are well maintained.

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INFLUENCE OF HOST FACTORS ON NEUROINVASIVENESS OF VESICULAR STOMATITIS VIRUS

III. Effect of Age and Pathway of Infection on the Character and Localization of Lesions in the Central Nervous System

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Plates 6 to 9

(Received for publication, October 16, 1937)

The experimental data of the preceding investigations (1, 2) suggested that the failure of older animals, which readily succumb after intracerebral injection of the virus, to exhibit any clinical manifestations of central nervous system (CNS) involvement after peripheral inoculations was possibly due to the presence of certain barriers to the invasiveness of the virus, encountered at the site of inoculation when the intramuscular or intraocular routes were employed and within the CNS when the virus was given by way of the nose. The hypothesis of localized barriers developing with increasing age of the host depends to a great extent on evidence that the distribution or movement of this virus in the nervous system of mice occurs by different mechanisms after peripheral and intracerebral inoculations. The purpose of the present investigation was to determine whether a detailed study of the localization of lesions in the nervous systems of susceptible and resistant mice after different modes of inoculation could throw additional light on the movement of the virus and thus on the nature of the resistance of older animals.

If the movement of vesicular stomatitis virus in the nervous system of mice occurs along the pathways suggested by the preceding experimental studies, one would expect that the distribution of the lesions would vary with intracerebral and peripheral inoculation, and that after peripheral injection they would also vary with the central connections of the nerves along which the virus gained

entrance. The successful demonstration of such specific variations in the localization of CNS lesions could add not only to the evidence for the axonal and transsynaptic movement of this virus but also to the hypothesis of localized barriers along such a path. If, on the other hand, the distribution of the lesions should prove to bear no relationship to the route of inoculation or not to depend upon the central connections of the neurons supplying the sites of peripheral injection, then the above concepts would become untenable.

Histopathological studies have been successfully employed by a number of investigators (Goodpasture and Teague (3); Marinesco and Draganesco (4); Pette (5); Hurst (6); Sabin and Hurst (7)) to show that with certain viruses the primary lesions in the CNS are determined by the peripheral nerve supply of the inoculated area. The relation of secondary and subsequent localizations of lesions in the CNS to the known central connections of the site of primary attack has been studied but little. Seifried and Spatz (8) recently stated that the spread of the viruses of poliomyelitis, "epidemic encephalitis," rabies, and Borna's disease within the CNS was by way of the spinal fluid. Fairbrother and Hurst (9) and Hurst (10) believed from their studies with the virus of poliomyelitis that both after intracerebral and intrasciatic inoculation the localization of secondary lesions within the CNS was determined by the tract connections of the site of primary attack. With equine encephalomyelitis, however, Hurst (11) stated that in the guinea pig "lesions were substantially the same whether the virus was introduced intracerebrally, intramuscularly, subcutaneously or intradermally," and in a subsequent communication (12), that the localization within the CNS was frequently essentially the same after intranasal as after intramuscular inoculation.

Methods

The mouse is particularly suited for this type of study because (*a*) its size readily allows sectioning of almost the entire CNS, and (*b*) in an investigation such as this it is important to determine not only that lesions are present in certain definite regions but also that they are absent in others.

In the beginning most of the work was done with brains taken out of the cranial cavity and fixed in Zenker's fluid containing 5 per cent glacial acetic acid. Later on, it was found that satisfactory fixation occurred through the bone, so that the entire head minus the skin and lower jaw was fixed in the Zenker-acetic mixture for 24 hours. The acid caused sufficient decalcification to permit the sectioning of all the structures, including the nasal mucosa, eyes, and cranial ganglia, *in situ*. In a few instances sections were cut parasagittally, extending from the nose to the medulla. As a rule, however, the entire skull was embedded (paraffin) with the anterior end down, the sections being cut serially in the frontal transverse plane, at a thickness of 5 to 6μ. Ribbons of four to six sections out of each twenty to thirty were taken for staining. The entire spinal column was fixed in the same manner so that sections at various levels showed not only the cord, roots, and

spinal ganglia but frequently also the sympathetic ganglia. The sections were stained with phloxine and methylene blue, eosin and methylene blue, or hematoxylin and eosin.

Remarks on Spontaneous Encephalitic Lesions in Mice

Pathological investigations on the CNS of rabbits were often confusing until the occurrence of spontaneous, asymptomatic, encephalitic lesions generally referred to as being caused by *Encephalitozoon cuniculi* was recognized. That similar difficulties complicate studies on the CNS of mice has not as yet been sufficiently appreciated. Cowdry and Nicholson (13) found meningoencephalitic lesions in the brains of twenty-five of 132 mice studied, and in five of the twenty-five positive cases they noted the coexistence of protozoan-like parasites. Smadel and Moore (14), in their report on the pathology produced by the virus of St. Louis encephalitis in mice, also noted the presence in some of their animals of these spontaneous encephalitic lesions. In the present investigation it was observed that the occurrence of spontaneous meningoencephalitic changes in the Rockefeller Institute mice was distinctly related to the age of the animal; while none was detected in about fifty young mice (less than 1 month old), practically all the old animals (about 9 to 12 months of age) showed these lesions when many sections of the same brain were examined. The pathology consisted of dense perivascular cuffs of small, round cells, meningeal infiltration with similar cells, and small glial and occasionally granulomatous foci in the brain substance. These lesions could be found scattered irregularly throughout the CNS from the tips of the olfactory bulbs all the way down to the sacral cord. Sometimes, however, many sections of the same brain had to be examined before one or the other of these changes was found. Although no protozoan-like parasites were observed in any of the present sections, one cannot exclude the possibility of their having been in the brains at some previous time. In view of the fact, however, that the spontaneous virus encephalomyelitis described by Theiler (15) is endemic in the Rockefeller Institute stock (1),[1] one must consider the possibility that some of these chronic meningoencephalitic reactions (particularly those which do not show granulomatous foci) may, perhaps, represent the residual lesions of a subclinical attack with this virus.

General Aspects of Vesicular Stomatitis Virus Lesions

In the CNS, primary necrosis of nerve cells is the outstanding lesion. The process is acute and the reaction to it consists chiefly of an invasion of leucocytes into the necrotic zones. Perivascular cuffing is either absent or extremely rare and inconspicuous; only occasionally does one find a vessel surrounded by a single layer of polymorphonuclear and mononuclear cells. There is no definite evidence

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[1] In the past 2 years the incidence of the apparent form of this type of encephalomyelitis was found to be 1 or 2 per 1000.

of a direct attack of the virus on the meninges after intracerebral or peripheral inoculation. There is, however, some infiltration of the meninges with mononuclear and polymorphonuclear cells, representing a response to injury of the nervous tissue, although the distribution of the exudate bears no particular relationship to the presence or absence of cerebral changes in a particular region. There is no difficulty in distinguishing the rather mild, acute, inflammatory reaction in the virus lesions from the diffusely scattered, chronic, and intense perivascular and meningeal infiltration encountered in the spontaneous cases. Small, round, acidophilic, intranuclear "inclusions" have been described as occurring in nerve cells affected by vesicular stomatitis virus (16). In the present study these were found so rarely as to be of no practical aid in mapping out sites affected by the virus. A more frequent change preceding the complete disintegration of the cell consists of a marked increase in the nuclear acidophilic material which shrinks from the membrane, giving rise to a clear halo. The nucleolus, however, remains *in situ*, and there is no margination of the basophilic material. This picture resembles nerve cell changes in mice injected with yellow fever virus (17), and with other viruses in nerve cells of other hosts, but does not appear to us to have the characteristics of a specific inclusion body.

Pathology after Intracerebral Injection

Young Mice.—The CNS of three mice, 15, 21, and 30 days of age, were examined. Mice of this age die within less than 48 hours after intracerebral injection of 100 or more minimal infective doses. When many sections are made the site of inoculation can be recognized as a tract of necrotic cells extending through the neopallial cortex and into the underlying diencephalon or mesencephalon. The lesion is sharply demarcated and appears to correspond roughly to the size of the injecting needle. From the site of inoculation, lesions are found to extend anteriorly and posteriorly in close relation to the ventricular system and its extension anteriorly, the rhinocele, and posteriorly along the ependyma of the central canal.

The brain of the 15 day old mouse showed no changes anterior to the lateral ventricles. In the other brains, the olfactory bulbs exhibited extensive necrosis, most marked in and about the rhinoceles and involving the cells of the internal granular and mitral layers. The outer layers of the bulbs (external granular, glomerular, and layer of nerve fibers) appeared generally well preserved, suggesting that the spread of the lesions was from the rhinocele outwards. Longitudinal and partial transverse serial sections indicated that the lesion was a continuous one, extending from the lateral ventricles to the tips of the olfactory bulbs. At the level of the lateral ventricles there was evidence of involvement of the ependymal cells as well as necrosis of a few layers of periventricular nerve cells, more marked ventrally but occurring also at the sides and dorsally in the corpus callosum. Anterior to the optic chiasm there were no other discernible lesions and the meninges showed no evidence of being attacked by the virus or of having permitted it to pass through it to the underlying brain tissue. Beyond the level

of the optic chiasm the periventricular distribution of the lesions was again apparent. There was necrosis of the cornu Ammonis where it forms the floor and inner sides of the lateral ventricles, as well as of the parts of the neopallial cortex which form the roof and outer sides. These changes did not extend for any appreciable distance beyond the ventricular walls. With the exception of a few necrotic cells and some polymorphonuclear leucocytes in the habenular nuclei, there was little involvement of the tissues surrounding the third ventricle. In one case (15 day old mouse) there was a considerable invasion of tissues around the third ventricle with polymorphonuclear leucocytes but without any evidence of cellular necrosis. Again with the exception of a small area of necrosis in the tectum of one brain, apparently forming a part of the needle tract, there were no significant lesions in the midbrain.

The meningeal reaction was slight in two instances, consisting chiefly of the infiltration of polymorphonuclear and a few mononuclear leucocytes, and rather marked in the third which exhibited an exudate containing a great deal of fibrin and polymorphonuclear leucocytes, particularly around the midbrain and in the region of the dorsal portion of the third ventricle. After intensive study this severe reaction appeared to be due neither to an attack of the virus on the meninges themselves nor on the underlying brain tissue, but rather to the trauma of inoculation.

Continuing posteriorly, one found an occasional necrotic focus in relation to the fourth ventricle, and definite involvement of the ependyma of the central canal and the contiguous nerve cells. Strangely enough the lesion was very slight in the cervical and thoracic spinal cord, but quite extensive in its lumbar portion.

No lesions were found in any of the following structures connected with the CNS: the sensory ganglia of the cranial nerves, the spinal ganglia, the submaxillary, ciliary, otic, and superior cervical sympathetic ganglia, the hypophysis, pineal body, retina, and nasal mucosa.

In summary it can be stated that after intracerebral injection of vesicular stomatitis virus in young mice the recognizable CNS lesions present shortly before death of the animal are situated along the ventricular system and its extensions in the brain, and along the central canal of the spinal cord. The indications are that the primary spread of the virus occurred along this open pathway. There is no evidence that the meninges are attacked by the virus (not even at the site of inoculation), nor that virus spreads along the subarachnoid space.

Old Mice.—It has already been indicated that old mice (about 8 months to 1 year of age) and young mice are equally susceptible to intracerebral inoculation of vesicular stomatitis virus in the sense that the minimal infective dose is the same

for both, but that the old mice develop signs and succumb a day or two later than the young ones. The brains of two intracerebrally injected, 1 year old mice, sacrificed when prostrate and near death, were studied. The CNS of the mouse sacrificed 4 days after inoculation showed no lesions which could be attributed to the effect of the virus. The changes present corresponded entirely to those of "spontaneous encephalitis" encountered in uninoculated old mice. The CNS of the mouse sacrificed 3 days after inoculation also showed the lesions of spontaneous encephalitis with several granulomatous nodules in one olfactory bulb and widespread lymphocytic perivascular and meningeal infiltration, but there were, *in addition*, two slight and very sharply limited lesions of the type *encountered in the young mice. One of these was in one of the olfactory bulbs* and consisted of an area of acutely necrotic nerve cells infiltrated with polymorphonuclear cells, extending from the rhinocele up to and including a few cells of the mitral layer. Sections of the same olfactory bulb just anterior or posterior to this area appeared entirely normal. The other zone was ventral and lateral to the fourth ventricle and consisted of a few necrotic nerve cells and a few polymorphonuclear cells.

It appears from these data that the nerve cells of the old mice are generally more resistant to necrosis than are those of the young animals, in spite of the fact that sufficient multiplication of virus and change occur in these cells to give rise to signs of encephalitis and death. This relative absence of significant lesions is even more remarkable since the infective process lasts twice as long in the old as in the young mice.

Pathology after Nasal Instillation of Virus

Young Mice.—Nine mice (seven 15 days and two 21 days old) were studied. Mice of this age succumb 4 or 5 days after nasal instillation of virus. Two 15 day old animals were sacrificed 2 days after instillation when they still appeared entirely well. The sections through the nasal mucosa did not permit any definite conclusion about the state of the cells in the olfactory or respiratory mucosa. There was, however, neither necrosis nor inflammation. The olfactory nerve roots in the mucosa and at their junction with the olfactory bulbs showed no inflammatory or other visible change. The meninges over the olfactory bulbs and the rest of the brain appeared entirely normal, as did the nerve tissue itself, with the possible exception of some of the mitral cells in the olfactory bulbs. These cells showed what may perhaps represent the early changes of virus action.

The material from one of the remaining seven mice was obtained immediately after death, while the others were sacrificed either when they showed pronounced nervous signs or when prostrate and near death. While there was a certain amount of individual variation in the extent and location of lesions, their dis-

TABLE I

Distribution of Necrotic Lesions in Various Regions of the Central Nervous Systems of Mice Succumbing after Nasal Instillation of Vesicular Stomatitis Virus

Region	Age of mice and remarks					
	15 days old			21 days old		1 yr. old
	Dead 4½ days (no circling)	Sacrificed 4th day (circled left)	Sacrificed 4th day (no circling)	Sacrificed 5th day (circling)	Sacrificed 5th day (no circling)	Sacrificed 8th day (no circling)
Respiratory mucosa	−	−	−	n.s.	n.s.	n.s.
Olfactory mucosa	++	+++	+++	n.s.	n.s.	n.s.
Olfactory bulbs	+++	++++	++++	+++	+++	++
Lateral olfactory gyrus	−	+	++++	−	±	+ (unilateral)
Septum (ventromedial aspect)	−	−	++	−	−	+++
Tuberculum olfactorium	+++	+++	+++	+++	++++	+
Piriform lobe (including amygdaloid complex)	−	−	+++	−	−	++ (unilateral)
Cornu Ammonis	−	−	++	−	−	+
Hypothalamus (tuber cinereum)	+++	+	+++	++	+++	+
Thalamus	−	−	?	−	−	±
Mammillary body	+	+	+	+	+	±
Habenular nuclei	−	−	++	−	+	±
Interpeduncular nucleus	−	−	++	−	+	+
Lateral and medial geniculate bodies	−	−	−	−	−	−
Superior and inferior colliculi	−	−	−	−	−	−
Tegmental nuclei, anterior	± (red nucleus)	−	−	−	−	+++
Gudden's and other posterior tegmental nuclei	+++	+	+++	++	+++	++++
Nuclei of pons	−	−	−	−	−	−
Neopallial cortex	−	−	−	−	−	−
Corpus striatum	−	−	−	−	−	−
Sensory nucleus of fifth nerve	−	−	−	−	−	−
Motor nucleus or fifth nerve and reticular formation	+	+	++	+	−	+++
Vestibular nucleus of eighth nerve	−	++ (left side)	−	++	−	−
Deiters' and Bechterew's nuclei	−	++ (left) side)	−	+	−	−
Cerebellum—deep nuclei	−	−	−	+	−	−
—rest of medula and cortex	−	−	−	−	−	−
Spinal cord	−	−	n.s.	n.s.	±	−
Gasserian ganglia	−	−	−	n.s.	n.s.	n.s.
Superior cervical sympathetic ganglia	−	−	−	n.s.	n.s.	n.s.
Submaxillary ganglia	−	−	−	n.s.	n.s.	n.s.

n.s. = no sections.

± = necrosis of occasional cell, found with difficulty.

+ = small necrotic focus present in only a few of a series of sections from the same region.

++ = limited zone of necrosis present in most sections of series from same region.

+++ = extensive necrosis seen in all sections of region.

++++ = almost complete necrosis of region.

− = no evident neuronal lesion.

tribution corresponded to so definite a plan that they can all be described together. Some idea of the variations can be obtained from Table I, in which the presence or absence of lesions in certain regions of the CNS of five young mice is indicated.

The olfactory mucosa constitutes the greater portion of the nasal membrane of mice and it appeared to be primarily attacked by the virus. The lesion manifested itself as a necrosis of scattered patches of olfactory mucosa with little or no inflammatory response. At this stage many areas were denuded of cells, the necrotic debris lying free in the nasal cavities. No definite evidence could be found of involvement of the respiratory mucosa, although the occasional presence of suspicious intranuclear acidophilic dots was somewhat confusing. No lesion of any kind was found in the roots of the olfactory nerve in the mucosa, nor as a matter of fact in the outer layer of the olfactory bulbs consisting of the lamina fibrorum nervi olfactorii. The chief lesion in the olfactory bulbs was in the mitral cell layer, where a varying number or practically all the cells may be necrotic. Frequently there were only empty spaces left where the mitral cells had been present originally. Depending upon the severity of the process, there was necrosis of the cells in the internal granular and gelatinous layers, and only rarely in the cells of the external granular layer surrounding the glomeruli which, as a rule, were entirely spared. The distribution of lesions in these olfactory bulb sections was quite different from that seen after intracerebral injection. After nasal instillation of virus the mitral cells appeared to be the center of the lesions and, even when practically the entire bulb was necrotic, the rhinocele remained undamaged, while after intracerebral injection it was clear that it was from this structure that the lesion extended.

Beyond the olfactory bulbs where the neopallial cortex joins the rhinencephalon, the former appeared entirely normal and in the latter there was usually a zone of varying extent which showed no lesions until the region of the tuberculum olfactorium and anterior perforated space was reached. Here there was a varying amount of necrosis in every case, while the lateral olfactory gyrus showed definite necrosis in only one and involvement of a very small patch of cells in two others; the septal region was involved in only one case. There were no lesions whatever around the rhinocele or the lateral ventricles, nor was there necrosis of tissue adjacent to the meninges. The lesions just described were practically always separated from either the ventricles or the meninges by normal appearing tissue.

Posterior to the optic chiasm the further distribution of necrotic foci appeared to depend upon the position of the lesions anterior to it. Thus, the cornu Ammonis was unaffected in all but one instance, and in that instance there was also necrosis in the ventromedial aspect of the "septum." Again, in only one case was there necrosis in the piriform lobes, and then it was unilateral and on the same side as extensive involvement of the lateral olfactory gyrus. The tuber cinereum of the hypothalamus was affected in all mice and some slight necrosis could always be found in the mammillary body. The habenular nuclei showed foci of necrosis in two mice. No lesions were found in the thalamus or other diencephalic structures. The tectum of the midbrain was negative in all cases, as was the anterior

portion of the tegmentum, with the possible exception of a few necrotic cells in one red nucleus of one brain. The interpeduncular nucleus showed necrosis in two cases and it is to be noted that it occurred only in the brains with involvement of the habenular nuclei. There were always, however, lesions in the posterior part of the tegmentum in the region of Gudden's nucleus and to a varying extent ventrally in the nuclei of the raphé and occasionally laterally, involving the mesencephalic nucleus of the fifth nerve. The nuclei of the pons exhibited no lesions. In the medulla there was necrosis in one or both motor nuclei of the fifth nerve and in varying regions of the formatio reticularis in all but one case.[2] There was never any evidence of involvement of the sensory nucleus of the fifth nerve. In two instances which corresponded with circling as the outstanding clinical sign, there was unilateral necrosis in the area of the vestibular nucleus of the eighth nerve, and the region of Deiters' and Bechterew's nuclei. In one of these brains, the nuclei of the cerebellum showed necrosis, while in all other instances the cerebellum appeared normal. The neopallial cortex was never involved. The spinal cord was examined in three cases and, with the exception of a few polymorphonuclear and necrotic nerve cells, there were no significant changes.

Special attention was paid to the nuclei of the other nerves supplying the nasal mucosa but no lesions were found in the Gasserian (sensory fifth), submaxillary ("parasympathetic"), or superior cervical (sympathetic) ganglia. It may also be stated that lesions were seen neither in the other sensory or autonomic cranial ganglia nor in the spinal ganglia. The lungs, liver, spleen, kidneys, and suprarenals of mice sacrificed on the 2nd and 4th days of the disease showed no changes attributable to the action of the virus.

Relation between Central Connections of Nerve Supply of Nasal Mucosa and Distribution of Lesions.—From the foregoing description, it is evident that of the various nerves connected with the nasal mucosa (sensory fifth, sympathetic and parasympathetic fibers, and olfactory nerves[3]) only the olfactory pathway showed signs of having been traversed by the virus. The choice of this special pathway by vesicular stomatitis virus cannot be regarded merely as a natural consequence of special anatomical relations (the large number of exposed olfactory neurons and possibly other direct connections with the CNS), since, as will be shown in another communication, not all viruses given intranasally to mice of the same breed and age invade the CNS along the olfactory nerves, but select instead the other nerves of the nasal mucosa. The use of the olfactory pathway should, perhaps, be regarded, therefore, as the result of some special affinity of vesicular stomatitis

[2] With regard to the involvement of the various regions in the midbrain, pons, and medulla reference may be made to Wallenberg's studies on the ramifications of the basal olfactory tract or bundle in the rabbit. (Wallenberg, A., *Anat. Anz.*, 1901–02, **20**, 175.)

[3] This does not include the small nervus terminalis about which relatively little is known and which was not readily distinguished in our sections.

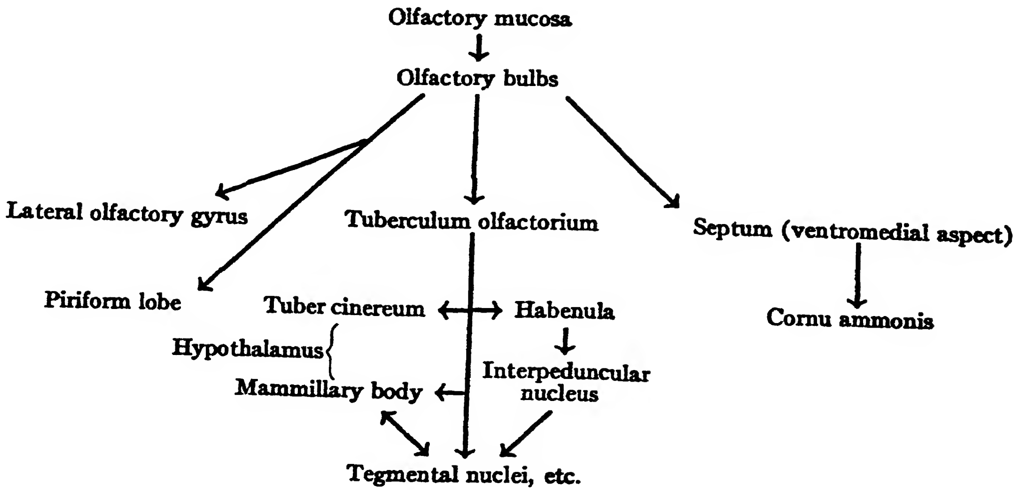
virus for the olfactory neurons in the nasal mucosa. These neurons are in synaptic relation with the mitral cells in the olfactory bulbs, the synaptic junction occurring not directly on the bodies of these neurons but rather at some distance on the dendrites entering the glomeruli. It is significant, therefore, and perhaps indicative of the mode of virus progression that one can find necrosis of the olfactory mucosa with no visible change in the olfactory nerve fibers, glomeruli, and the susceptible cells of the external granular layer which surround them, and yet see almost complete destruction of the mitral cells beyond. In an advanced lesion there is, of course, necrosis of granular cells (they are connected with the mitral cells), particularly of the internal layer, but even then most of the glomeruli and the surrounding cells remain intact.

The axons of the mitral cells grouped in the lateral, intermediate, and medial olfactory striae are said to terminate, either directly or after preliminary synapse in the anterior olfactory nucleus, chiefly among the neurons of the lateral olfactory gyrus, tuberculum olfactorium (region of anterior perforated substance), and questionably in the septum,[4] and it is in these areas also that the further localization of lesions occurs. *It is noteworthy that the olfactory bulb lesions are not continuous with those further posteriorly, i.e., that there is between them, almost invariably, an apparently undamaged zone.* An analysis of the subsequent localization of lesions (Table II) indicates that the regions involved depend upon the major central connections of olfactory areas which have been previously affected. Thus, in accord with the constant involvement of the intermediate olfactory nucleus (anterior perforated substance, tuberculum olfactorium), one finds always affected the hypothalamus (tuber cinereum and mammillary body), and tegmental nuclei (constituting one of the descending olfactory correlation pathways). Occasionally the pathway through the habenular nuclei and the interpeduncular nucleus is used. In view of the fact that the neurons in the interpeduncular nucleus participating in the olfactory relay are in relation proximally only with cells in the habenular nuclei, it should be noted that when lesions are observed in the interpeduncular nucleus they are invariably also found in the habenular, even though they may be so limited as to require many sections to disclose them. The cornu Ammonis was involved but once and that occurred in a brain which showed necrosis in the ventromedial, septal region anterior to the optic chiasm. The piriform lobes also showed necrosis in only one case and that in association with a lesion of the lateral olfactory gyrus on the same side. Why the tuberculum olfactorium or anterior perforated substance should be so constantly involved, while the lateral olfactory gyrus, piriform, and septal regions

[4] Any simple or short description of the central connections of the olfactory neurons of the second order is necessarily incomplete. The termination of such fibers in the septum of higher mammals is especially open to question. The occasional presence of necrosis in the ventromedial aspect of the septum (parolfactory area, paraterminal body) (see Fig. 6) in some of these mice should, therefore, be interpreted with that in mind.

TABLE II

Scheme Indicating Order of Distribution of Neuronal Lesions after Nasal Instillation of Virus



The purpose of this scheme is to show how the localization of lesions deep within the CNS occurred in accord with the involvement of certain definite, intermediate zones. Thus there were no lesions in the interpeduncular nucleus without involvement of the habenular nuclei and tuberculum olfactorium; none in Ammon's horn without damage to the septum, etc.

are only occasionally affected, is not clear. According to the view of axonal and trans-synaptic progression of the virus, it could perhaps be accounted for if the axons of the greater number of mitral cells were included in it, or if the greater number or the more suitable synaptic connections were in this relay. The diencephalic and tegmental mesencephalic structures which are most frequently affected are the ones which are chiefly concerned in the correlation of olfactory with other impulses and in the function of relay stations on a reflex pathway to the motor and perhaps other nuclei of the hind-brain.

Findings in Old Mice Showing No Clinical Signs of Disease.—Nine mice (eight were approximately 1 year old and one was 31 days old) were studied at various intervals after nasal instillation of virus. Two were sacrificed on the 2nd day, two on the 4th, and one on the 5th, 6th, 7th, 10th, and 14th days, none showing any signs of disease. In none of these animals did the CNS show any changes which could be attributed to the effect of the virus. No definite effect ascribable to the action of the virus on the nasal mucosa was demonstrated. In some of the mice the presence of large numbers of pus cells in the sinuses, tissues, and nasal cavities was perplexing until the same picture was observed in two "normal" old mice which received no virus. No definite conclusion could be reached about the significance of occasional intranuclear, acidophilic bodies.

The absence of obvious lesions in these mice, in spite of the fact that virus can be demonstrated in the rhinencephalon anterior to the optic chiasm between the 2nd and 5th days, is not surprising in view of the fact that they may be practically absent in old mice succumbing with encephalitis after intracerebral injection of the virus.

Findings in Old Mice Showing Signs of Encephalitis or Myelitis.—A varying number of old mice are not resistant to nasal instillation of the virus and develop, after a relatively prolonged incubation period of 8 to 14 days, either signs of encephalitis or merely flaccid paralysis of the posterior extremities. The CNS of two such mice were studied, and the extensive lesions which they exhibited were in marked contrast to the findings in the intracerebrally injected animals. One was sacrificed on the 8th day, when it showed signs of encephalitis for the first time. Lesions quite as extensive as those found in the young mice were present here and in the same regions (see Table I). It should be noted that these extensive lesions clearly due to virus action were present side by side with those of spontaneous encephalitis. The other mouse studied histologically was the only one of a group of eleven 1 year old, similarly instilled mice to develop nervous signs. These appeared in the form of flaccid paralysis of the posterior extremities on the 8th day after nasal instillation, and the mouse was sacrificed 9 days after the onset of paralysis. The striking fact here was that while there was rather marked destruction and neuronophagia in both lateral regions of the anterior horns of the lumbar cord, there was no evident involvement of either the ependyma

and periependymal tissue, the posterior horns of this level, or of any part of the cervical, thoracic, and sacral levels. While insufficient sections of the brain were available to determine the exact tracts by which the cord might have been reached, clearly defined foci of necrosis were found in the hypothalamus, thalamus, and habenular nuclei. It is noteworthy that the spinal ganglia, even of the lumbar level, showed no lesions, and also that there was neither meningeal nor perivascular reaction in the cord.

Pathology after Intraocular Injection

Young Mice.—15 day old mice uniformly succumb after intraocular (vitreous) injection of the virus and are almost as susceptible to inoculation by this route as by the intracerebral. The incubation period is considerably prolonged in 21 day old mice and many of them are completely resistant, even to the highest concentration of virus, while 1 year old animals, with only few exceptions, exhibit no clinical signs of disease whatever. It should also be recalled that while in susceptible mice, 21 days of age or older, it was possible to show by subinoculation methods that the primarv spread of the virus was probably along the optic nerve with an early localization in the contralateral diencephalon and mesencephalon, no conclusion could be drawn from the experiments with the 15 day old animals because the virus, when first demonstrated in the CNS, was already widely scattered (2). One of the questions, therefore, was whether the marked and uniform susceptibility of the 15 day old mice is due to a mode of virus progression different from that which obtains among the older animals, or whether the same pathways are pursued in all, the rate of progression being so much greater in the 15 day old mice that the entire nervous system is rapidly invaded by varying amounts of virus.

The pathology after intraocular injection was therefore studied chiefly in 15 day old mice. It was early observed that the CNS lesions in these animals could apparently be accounted for on the basis of primary virus progression only along the decussating optic nerve pathway. In each of three mice, for example, whose right eye was inoculated marked necrotic lesions were present in the left superior colliculus, while the right one appeared entirely uninvolved. To eliminate the possibility that the *left* superior colliculus may be particularly susceptible to the action of this virus and to establish more convincingly that the decussating pathway is used, the brains of two 15 day old mice injected into the left eye were studied. In these two animals the right superior colliculi showed marked necrosis, while the left ones remained uninvolved, thus indicating rather conclusively that in 15 day old mice the virus follows the decussating pathway suggested by the experimental observations on older animals.

A detailed tabulation of the localization of lesions in various regions of the CNS of each of the five mice is presented in Table III. Four were sacrificed 3 days and one 4 days after inoculation at a time when they showed advanced nervous signs or were prostrate. Sections of the inoculated eyes revealed in each instance

TABLE III

Distribution of Necrotic Lesions in the Central Nervous Systems of 15 Day Old Mice Succumbing after Intraocular (Vitreous) Injection of Virus

Region		Eye inoculated and day on which mice were sacrificed				
		Right eye			Left eye	
		3rd	3rd	3rd	4th	3rd
Inoculated eye	Retina	+±	++	++	++++	+
	Cornea, iris, etc.	–	–	–	–	–
Uninoculated eye		–	–	n.s.	–	–
Optic nerve near chiasm	Right	+	n.s.	n.s.	–	–
	Left	–	n.s.	n.s.	+	±
Optic chiasm and tract beyond decussation	Right	–	–	–	+	+
	Left	+	+	+	–	–
Lateral geniculate body	Right	–	–	–	+	–
	Left	+++	–	–	–	–
Medial geniculate bodies		–	–	–	?	–
Superior colliculus	Right	–	–	–	++++	+++
	Left	++++	+++	+++	–	–
Inferior colliculi		–	–	–	–	–
Region of oculomotor and trochlear nuclei		–	–	–	++ (right)	–
Tegmentum	Anterior (including red nuclei)	–	–	–	+++	–
	Posterior (to red nuclei)	?	–	++ (raphé)	+++	± (raphé)
Hypothalamus (contiguous to optic tract)		–	–	±	++ (right side adjacent to optic tract)	–
Thalamus		+ (left lateral nucleus)	–	–	–	–
Mammillary body		–	–	±	+	–
Habenular nuclei		–	–	–	–	–
Interpeduncular nucleus		–	–	–	–	–
Ganglion basale opticum	Right	–	–	–	–	–
	Left	±	±	±	–	–
Cornu Ammonis		–	–	–	–	–
Piriform lobes		–	–	± (left side adjacent to optic tract)	+ (right side adjacent to optic tract)	–
Septum	Right	–	–	–	–	–
	Left	–	–	–	–	–

See legends of Table I.

TABLE III—*Concluded*

Region		Eye inoculated and day on which mice were sacrificed				
		Right eye			Left eye	
		3rd	3rd	3rd	4th	3rd
Tuberculum olfactorium	Right	–	–	–	+	–
	Left	–	–	++	–	–
Lateral olfactory gyrus	Right	–	–	–	–	–
	Left	–	–	+++	–	–
Olfactory bulb	Right	–	–	–	++	–
	Left	–	–	+++	–	–
Corpus striatum		–	–	–	–	–
Neopallial cortex		–	–	–	–	–
Nuclei of pons		±	?	±	++	–
Medulla, reticular substance		–	–	n s.	+++	++
Cerebellum		–	–	–	–	–
Spinal cord		–	n.s.	–	n.s.	n s.

a lesion of varying extent in the retina with no evidence of any specific action on any of the other structures. In one mouse (sacrificed on the 4th day) more than one-half of the entire retina was destroyed, leaving not a trace of its original structure or cell outlines. In the other animals the retinae exhibited scattered foci of necrosis involving all the layers to a varying degree. Small numbers of polymorphonuclear and occasional mononuclear leucocytes infiltrated these foci and particularly the outer layers of nerve fibers and ganglion cells. Only a few of the invading cells were found free in the posterior or anterior chambers. The uninjected eyes appeared normal. Sections of eyes removed 4 days after inoculation of broth, or normal mouse brain suspension for control showed only a few polymorphonuclear and mononuclear cells in the posterior and anterior chambers but no changes whatever in the retina. Some inflammatory exudate in the outer coats and muscles of the eye apparently near the site of inoculation was found both after virus and control inoculations.

Examination of the optic nerve of the inoculated eye revealed no change except in the portion near the optic chiasm. This change, consisting of a varying degree of disorganization of the normal structure with necrosis of some of the large interstitial glial cells and infiltration with polymorphonuclear leucocytes, was also found in the middle of the optic chiasm and after the decussation on the side opposite to that of the injected eye. Pathological changes in the optic chiasm beyond the decussation were noted on the left side in mice, which received the virus in the right eye, and on the right in those inoculated in the left eye. In

some of the mice the same sort of change continued in the contralateral optic tract beyond the chiasm.

The one other lesion constantly exhibited by all the mice consisted, as already indicated, of extensive necrosis of the contralateral superior colliculus. In one mouse, sacrificed when prostrate on the 3rd day, this was practically the only finding apart from those already mentioned. Of the three mice which received virus in the right eye, two showed no lesions in the lateral geniculate bodies of either side, while in the third one only the left (*i.e.*, contralateral) lateral geniculate body was affected, showing extensive necrosis of both the dorsal and ventral nuclei with practically no infiltrating inflammatory cells. Here were found many neurons which exhibited the early cytoplasmic and nuclear changes which have already been described. In this mouse the same changes were also present in the left lateral nucleus of the thalamus. Of the two mice, in which the inoculation was made in the left eye, one showed distinct, though not as extensive, necrosis of the right lateral geniculate body with no perceptible involvement of the left one, and in the other there was no lesion in either. It was thus clear that the localization of the most obvious lesions depends on the distribution of the greater number of the axons of the retinal ganglion cells.

Two of the five mice exhibited significant unilateral lesions in the olfactory pathway. The chief interest of this involvement lies in the fact that in both cases it was the contralateral olfactory pathway which was affected. In one of these mice, whose right eye was inoculated, the right olfactory pathway appeared entirely normal, while moderately extensive foci of necrosis (not contiguous with one another) were present in the tuberculum olfactorium, lateral olfactory gyrus, and olfactory bulb of the left side. In the other mouse the virus was injected in the left eye, and the left olfactory pathway appeared normal, while the tuberculum olfactorium and olfactory bulb of the right side showed distinct foci of necrosis. It is apparent from this localization that these lesions cannot be explained on the basis of an escape of some of the virus from the conjunctival sac into the nose, for then they should have been on the same side as the inoculated eye. The explanation for the involvement of the contralateral olfactory pathway may, perhaps, be found in the changes spreading from the optic tract, beyond the decussation of the optic chiasm, to the structures with which it is intimately connected by contiguity, *i.e.*, the tuber cinereum, the ganglion basale opticum (an olfactory ganglion), and the piriform lobe. Varying numbers of necrotic cells were found in all these structures in the zones which were in contact with the necrotic portion of the optic chiasm and tract. These changes were least noticeable in the mice sacrificed on the 3rd day, but quite marked in the one killed on the 4th day. It is apparent, however, that the further distribution of lesions from these olfactory centers anteriorly or posteriorly is not by contiguity, but apparently axonal and trans-synaptic from one level or station to the next. The two mice just described also showed necrosis of a small number of cells in the mammillary body and extensive destruction of the ventral portion of the posterior tegmentum (*i.e.*, posterior to the level of the red nuclei).

In none of the four mice sacrificed on the 3rd day were there any perceptible lesions just ventral to the aqueduct of Sylvius (region of nuclei of third and fourth cranial nerves and Edinger-Westphal nucleus), nor in any other part of the anterior tegmentum, (*i.e.*, portion containing red nuclei). The mouse sacrificed on the 4th day (left eye injected) showed marked necrosis in the region of the nuclei of the 3rd and 4th cranial nerves on the right side and almost complete destruction of the anterior and posterior regions of the tegmentum. The pons showed a varying number of necrotic cells in three mice, and the medulla some foci in the reticular substance of two.

No lesions were found in any of the mice in the cornu Ammonis, corpus striatum, neopallium, cerebellum, and spinal cord. It should also be noted that no lesions were seen in the ciliary, Gasserian, submaxillary, and superior cervical sympathetic ganglia.

Old Mice.—It has already been shown that in old mice which are resistant to intraocular injection, no virus can be demonstrated in the CNS (2). The chief interest, therefore, was in determining the effect of the virus in the eye, where its progression is apparently held up. The eyes of two old mice sacrificed on the 4th day showed no visible lesions in the retina nor any more cellular infiltration than was found in eyes injected with normal mouse brain suspension for control. The optic nerves and CNS similarly revealed no change.

Pathology after Intramuscular Injection

Young and Old Mice.—Young mice receiving virus in the muscles of one leg develop flaccid paralysis, first of the inoculated extremity, and succumb with signs of an ascending myelitis. Old mice, on the other hand, exhibit no signs of disease after intramuscular injection of the largest amounts of virus (10^7 M.C.L.D.). In the young mice the virus has been shown to multiply at the site of inoculation and to invade the spinal cord by way of the peripheral nerves, while in old mice neither local multiplication nor invasion of the peripheral nerves or CNS was demonstrable (2). One of the questions which arose during these experiments was whether the local multiplication of virus in the young mice occurred in nervous structures or in the muscle and other non-nervous elements. Another question was whether the intramuscular injection of large amounts of virus in old mice had any effect on the muscle or nervous tissue which might be demonstrable histologically though not by animal passage.

To answer these questions, histological studies were made of the muscles and peripheral nerves of the inoculated legs of young and old mice at various intervals after injection. For control, young and old mice were given similar amounts of normal mouse brain suspension and the above mentioned tissues removed at the same intervals after inoculation. To aid in the localization of the site of inoculation, a small amount of powdered charcoal was added both to the virus and normal brain suspensions. In mice sacrificed on the 2nd day the reaction to the normal mouse brain-charcoal powder mixture consisted of an infiltration of the interstitial connective tissue with polymorphonuclear and mononuclear cells. The

number of the former cells diminished on the 3rd day, and on the 5th day the inflammatory exudate consisted almost entirely of mononuclear cells. The reaction was practically the same in both the young and old mice. There was no evidence of any involvement of the muscle or nerve tissue at the injected site, and sections of the sciatic nerves appeared normal.

In the old mice injected with virus there was no perceptible difference from the reaction to normal tissue just described, while in the young mice, on the other hand, there appeared definite evidence of a direct attack of the virus on the muscle tissue. At 2 days after injection (the period when considerable multiplication of the virus was demonstrated and successful muscle to muscle passage was carried out (2)), there was as yet no evident necrosis of the muscle fibers and no appreciable difference in the inflammatory reaction from that observed in the control animals. On the 3rd day, fragmentation and hyaline-like necrosis of a small number of muscle fibers became apparent and an increase in the inflammatory exudate consisting mostly of polymorphonuclear leucocytes, many of which appeared to be phagocytizing the fragmented muscle fibers. On the 5th day practically the same picture was seen but in an exaggerated form: A large number of muscle fibers was completely necrotic and the phagocytosis of muscle fragments by numerous polymorphonuclear leucocytes (which may perhaps be defined as myophagia) closely resembled the picture of neuronophagia in the nervous system. Hypertrophy and proliferation of the sarcolemmal nuclei formed another prominent feature of the reaction at this time. No inclusions were seen in any of the cells on the 2nd, 3rd, or 5th days and no evidence could be found of a direct attack of the virus on the interstitial connective tissue, blood vessels, or nerve trunks lying in the vicinity of necrotic muscle tissue. Longitudinal sections through the sciatic nerves of the inoculated legs showed no inflammatory reaction at any time nor any other perceptible abnormality on the 2nd and 3rd days; on the 5th day the appearance was suspicious of a fragmentation of a certain number of nerve fibers, but no definite conclusion is possible with the method employed.

These histological findings thus proved to be in accord with the experimental results (2) and further elucidated the nature of local virus multiplication at the site of intramuscular injection in the young mice.

The CNS of two mice (15 days old) injected with virus into the right leg were examined; one on the 4th day, when it exhibited only paralysis of the posterior extremities, and the other on the 5th day immediately after death. In the animal sacrificed on the 4th day the outstanding lesion was found in the lower lumbar cord, where it seemed to be confined to the neurons of the anterior horns. These cells were in various stages of necrosis and early neuronophagia was present. A few of the blood vessels were surrounded by one or two layers of mononuclear and polymorphonuclear cells. The meninges showed no evidence of virus attack. The spinal and sympathetic ganglia at the lumbar levels of the cord having considerable anterior horn cell involvement exhibited neither neuronal necrosis nor inflammatory reaction. It would appear that either the virus entered the cord entirely by the efferent axons of the anterior horn cells distributed to the muscles

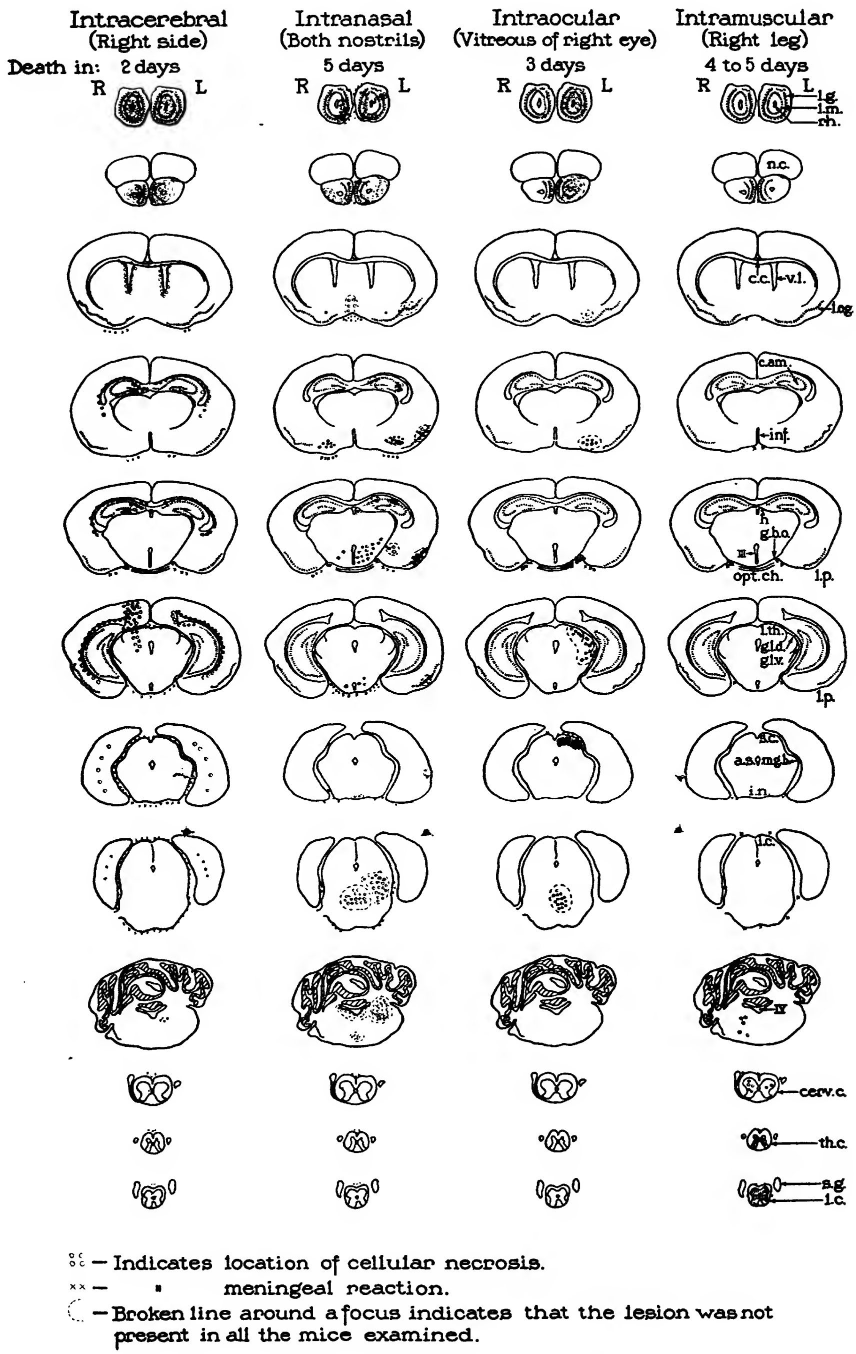
which it attacked, or if it also pursued the afferent sensory pathway, the neurons in the spinal ganglia must undergo necrosis much less readily than the motor cells. The thoracic and cervical levels of the spinal cord showed little change with the possible exception of some doubtful intranuclear inclusions in a few neurons. The only other neuronal lesions in the CNS were found in the ventral aspect of the medulla, slightly cephalad to the decussation of the pyramidal tracts, and particularly on the right side in the region of the lateral reticular and possibly of the olivary nuclei. Here there was evidence of early nerve cell necrosis, and some interstitial and perivascular infiltration with polymorphonuclear and mononuclear cells. A small number of these cells could be seen in the meninges of the ventral aspect of the brain as far cephalad as the olfactory bulbs.

In the second animal in which the infection was allowed to go on to a fatal termination, there was complete necrosis of almost all neurons, both in the anterior and posterior horns of the lumbar cord with very little inflammatory reaction. Thus neuronal destruction had already spread to most of the sacral and thoracic portions of the spinal cord, while in the cervical portion necrosis was still in its earlier stages. The only other nervous lesions noted were again in the reticular substance of the medulla, where foci of necrosis and neuronophagia could be found. The spinal ganglia appeared entirely uninvolved.

In summary it may be pointed out that the CNS lesions, after inoculation of the virus into the muscles of one leg, are distinctly different in distribution from those which follow intracerebral, intranasal, or intraocular injection of the virus. Their localization is in accord with a primary, insulated, axonal transmission of the virus, the most evident damage being observed not along the course of the axons but rather at the site of their cell bodies. The absence of lesions in the spinal ganglia, even at death, strongly suggests that the invasion of the virus into the spinal cord may have occurred chiefly along the efferent fibers supplying the affected muscles.

Correlation between Distribution of Lesions and Presence of Virus in the Central Nervous System

One is impressed with the fact that many areas in the CNS which were shown to contain virus by animal passage (2) exhibited no evident histological changes. This is particularly apparent among the 15 day old animals which were injected intraocularly. Thus, while animal passage revealed virus among other zones in the homolateral diencephalon and mesencephalon, and in the occipital cortex at an early stage, no lesions were found in these regions in any of the mice. In general, the impression is gained that in the case of vesicular stomatitis, virus multiplication precedes recognizable cytological changes by a day or two and that lesions appear where the virus is delivered first and perhaps in largest amount,



Text-Fig. 1. Influence of route of inoculation on CNS pathology in young mice (vesicular stomatitis virus, N. J.). Diagrammatic representation of localization of lesions in CNS of young mice after different routes of inoculation. The outlines of the cross sections of the brain and cord are about 4 times actual size. The lesions found in several mice inoculated by the same route were combined on one series of cross sections.

R, right side; L, left side; a.s., aqueduct of Sylvius; c. am., cornu Ammonis; c.c., corpus callosum; cerv. c., cervical cord; g.b.o., ganglion basale opticum (preoptic nucleus); g.l.d., dorsal nucleus of lateral geniculate body; g.l.v., ventral nucleus of lateral geniculate body; h, habenula; i.c., inferior colliculus; inf., infundibulum; l.c., lumbosacral cord; l.g., lamina glomerulosa of olfactory bulb; l.m., layer of mitral cells; l.o.g., lateral olfactory gyrus; l.p., lobus piriformis; l.th., lateral nucleus of thalamus; m.g.b., medial geniculate body; n.c., neopallial cortex; opt. ch., optic chiasm; rh., rhinocele (anterior extension of lateral ventricle); s.c., superior colliculus; s.g., spinal ganglion; th.c., thoracic cord; v.l., lateral ventricle; III. third ventricle; IV, fourth ventricle; i. n., interpeduncular nucleus.

death terminating the disease before the other areas, infected later and perhaps less heavily, have developed demonstrable changes. It does not appear that in young mice certain areas are more resistant to necrosis than others, because by varying the route of inoculation almost all regions of the CNS can be shown to be susceptible. Considering the extensive connections between various regions of the CNS, it is easy to understand how virus can spread almost simultaneously to many remote parts of the brain, but the localization of lesions chiefly in the zones which receive the greatest number of axons from neurons primarily infected is in good agreement with the hypothesis of axonal and trans-synaptic progression of the virus. In intracerebrally injected old animals, on the other hand, animal passage clearly demonstrated that virus had multiplied in widely scattered areas of the CNS within 24 hours after injection (1) and yet in moribund mice sacrificed on the 3rd and 4th days recognizable lesions were either entirely absent or involved only a small number of isolated cells. This observation indicates that at least in some of the older animals the cells have become distinctly more resistant to necrosis resulting from virus multiplication. It is to be recalled, however, that while the cells retain their essential structure and show no other changes recognizable by the methods employed, there is, nevertheless, impairment of their function as evidenced by the clinical signs of encephalitis and death of the animal. Further study may reveal that the resistance of nerve cells of old mice to necrosis may not be limited to vesicular stomatitis virus. In describing the pathology of yellow fever encephalitis in two mice, one 6 days old and one adult, Goodpasture (17) noted that in the young mouse there was a great deal of neuronal necrosis with almost imperceptible inflammatory reaction, while in the adult mouse despite the abundant perivascular and diffuse cellular exudation, neuronic alterations were difficult to find. While the inflammatory reaction in the old animal may have occurred in response to imperceptible virus action on the cells, it resembles very closely the picture of spontaneous encephalitis.

SUMMARY AND DISCUSSION

It will be well to restate the main problem at this point and to examine how far the accumulated data can help to elucidate it. The problem is this: Why are old mice generally resistant to all forms of peripheral inoculation of vesicular stomatitis virus when intracerebral injection is equally fatal for mice of all ages? The results of experiments in which the presence of virus was demonstrated by animal passage suggested that the reason can perhaps be found in (*a*) the different mechanisms of virus progression after intracerebral and peripheral injection, and (*b*) the development with age of localized barriers capable of halting the spread of virus (1, 2). The present study sought histological evidence for the nature of virus progression

and for the changes observed in the older animals. The results clearly demonstrate that after intracerebral injection virus spreads along an open system, the lesions being distributed almost entirely in contiguity with the ventricles and their extensions, while after peripheral inoculations the evidence points to progression of the virus in a closed system of neurons and their processes, at least in the stage preceding neuronal necrosis, the distribution of lesions depending upon the central connections of the primary neurons connected with the inoculated site. Thus, in young mice, nasal instillation of the virus was followed by necrosis of a long chain of neurons, starting with those in the olfactory mucosa and progressing through specific zones of the olfactory pathway, pursuing the same order in which the various regions are known to have their major connections with one another. It is important to note that after nasal instillation the apparent lesions were present where the cell bodies of the neurons are situated, and not along the tracts connecting one group of neurons with another, which accounts for the lack of contiguity between the affected zones and the normal appearing, intervening areas. The assumption that the primary progression of the virus in this case occurs in a closed system is based on the absence of lesions in unrelated areas contiguous to those which are necrotic and to the tracts which connect one affected zone with another.

Additional evidence for the assumption that the initial dissemination of peripherally injected virus is in a closed system is found in the decussating optic nerve pathway primarily pursued by the intraocularly injected virus. The progression of the virus along this decussating pathway was indicated in the experimental data obtained on mice 21 days or older, while in younger animals the spread of virus was so rapid and diffuse that the pathways along which it might have occurred remained obscure (2). In the present study, in which 15 day old mice were used, the lesions in the retinal neurons and the constant involvement of only the contralateral superior colliculus left little doubt that the *primary* spread of the virus, even in these very young animals, must have occurred within the retinal neuron processes (axons) which decussate in the optic chiasm (in the mouse, as in the rat, very few of these go to the homolateral side) and synapse chiefly with the neurons of the contralateral superior colliculus and

also, apparently to a lesser extent, with those of the contralateral external geniculate body, where lesions were also demonstrated. Virus spreading in the optic nerve along the perineural subarachnoid space would be found at the base of the brain at the optic chiasm; virus extending along the interstitial spaces in the optic nerve should involve not only the nuclei of both sides of the optic pathway but also non-optic structures, such as the medial geniculate bodies, posterior colliculi, etc., by means of the commissures of von Gudden and of Meynert, whose fibers course through the chiasm. The highly specific localization observed in the present study is best accounted for by progression along the suggested closed pathway. Hurst (10) observed that poliomyelitis virus, after injection into the left sciatic nerve, may, after invading the lumbar cord, be found first in the contralateral motor cortex or thalamus and he suggested that this was evidence of progression along a decussating pathway and in favor of the axonal hypothesis of virus spread. It was not shown, however, that this particular localization was specifically related to the introduction of virus in the left sciatic nerve, or that it could be reversed by inoculating the sciatic nerve of the opposite side. The hypothesis proposed by Hurst, however, finds support in the present instance for (*a*) the superior colliculi never showed lesions after intracerebral, intranasal, or intramuscular inoculations, and (*b*) necrosis was produced in either the right or the left superior colliculus, depending on whether the virus was injected into the left or right eyes.

The localization of lesions after injection of virus into the muscles of one leg indicated that in the young the invasion occurred along the local peripheral nerves, especially the motor fibers (neurons destroyed in the lumbar cord with those in the spinal ganglia intact), after a primary attack on the muscle itself. The only other lesions found at a late stage were in the reticular substance of the medulla, the olfactory portions of the brain appearing entirely normal. In this respect the mechanism of progression of intramuscularly injected vesicular stomatitis virus differs from that of eastern equine encephalomyelitis and pseudorabies viruses similarly injected into mice of the same age and breed: the former (E.E.E.) invades the central nervous system in the majority of instances, by being eliminated on the nasal mucosa and then along the olfactory pathways (18), while the latter

appears to employ chiefly the local sensory fibers, attacking primarily the neurons in the spinal ganglia (unpublished observations).

Because the CNS of old mice remain for the most part susceptible to vesicular stomatitis virus (although definite evidence of resistance to necrosis of the neurons was observed), and because after intracerebral injection the virus has been shown to spread in an open (ventricular) system, it is clear why young and old mice are equally susceptible to inoculation by this route. After peripheral inoculation, however, it has been amply demonstrated by experimental and histological methods that the spread of this virus begins and continues, at least until the cells disintegrate, in a closed system within the neurons and their processes and apparently also across the synapses. The halting of the virus somewhere in the anterior rhinencephalon after nasal instillation in resistant mice (1) would appear to be due to an arrest in an insusceptible neuron or an impenetrable synapse somewhere in the chain, and to the failure of the affected neurons to disintegrate (no lesions were found in the CNS of these mice) and thus to liberate the virus into the open system. After intramuscular injection, on the other hand, the virus encounters a different kind of muscle cell in the old mouse, and its inability to invade the nerves may perhaps be bound up with its demonstrated inability to attack and multiply in these changed muscle cells, although the rôle of a possible alteration in the terminal nerve endings themselves is not yet clear. After intraocular injection, the virus fails to affect visibly the retinal neurons of resistant old mice and the further invasion of the CNS is inhibited. The resistance of old mice to peripheral inoculations of vesicular stomatitis virus thus appears to be the result of (*a*) changes produced by age not in the whole animal but in certain specific, isolated structures, and (*b*) the special mode of progression of peripherally injected virus.

It may be of interest to point out two phenomena which may perhaps be related to the one investigated in the present study. Tobacco mosaic virus has been found to produce different types of disease in certain plants of different ages; thus a widespread, systemic necrosis leads to the death of young *Nicotiana rustica* plants, while in old plants it is possible to produce necrotic foci in many parts of the plant by direct inoculation, although generalization does not

occur from an isolated focus as it does in young specimens (19). In other words, age apparently does not change the whole plant, but it does transform something which allows the virus to spread easily from one site to another. MacNider (20) has observed that dogs which survive a severe type of hepatic injury from uranium, repair this injury with a special type of atypical, epithelial cell and become resistant not only to secondary intoxications by uranium but also by chloroform; he has also found that this change in epithelial cell type may be acquired as a product of senility, and that when it develops it imparts to the liver a degree of resistance to chloroform comparable to that induced by a process of repair following a severe hepatic injury from uranium nitrate.

CONCLUSIONS

The resistance of old mice to peripheral inoculations of vesicular stomatitis virus appears to be the result of (*a*) changes produced by age not in the whole animal but in certain specific, isolated structures, and (*b*) the special mode of dissemination of peripherally injected virus.

The equal susceptibility of young and old mice to intracerebral inoculation can be explained by the observation (*a*) that virus injected in this way spreads primarily in an "open system" (the lesions being distributed almost entirely in contiguity with the ventricles and their extensions), and (*b*) that in the old animals the central nervous system as a whole, although more resistant to neuronal necrosis, is still lethally affected by the multiplication of virus.

After peripheral inoculations the evidence points to progression of the virus in a closed system of neurons and their processes, at least in the stage preceding neuronal necrosis, the distribution of lesions in the central nervous system depending upon the central connections of the primary neurons connected with the inoculated site. In old mice the inability of the virus to involve the terminal processes or cell bodies of the neurons at the site of inoculation (as after intramuscular or intraocular injections) or to spread to other neurons along the chain in the central nervous system (as after nasal instillation) prevents the development of the clinically apparent and fatal disease.

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EXPLANATION OF PLATES

The sections in the plates are of material obtained from 15 day old mice and stained with phloxine and methylene blue.

Plate 6

Fig. 1. 4 days after injection of virus into vitreous humor of left eye. A, uninoculated eye; note normal appearance of retina. B, inoculated eye; arrows point to necrosis of retina. × 28.

Fig. 2. Necrosis of *right* superior colliculus after inoculation of *left* eye. × 31.

Photographed by Joseph B. Haulenbeek and Louis Schmidt

Plate 7

Fig. 3. Necrosis of *left* superior colliculus after inoculation of *right* eye. × 31.

Fig. 4. 4 days after nasal instillation of virus. Note necrosis and desquamation of part of olfactory mucosa. Left olfactory bulb shows extensive necrosis in all layers except the outermost one of nerve fibers; arrows point to almost complete disappearance of mitral cells. The right olfactory bulb shows a much earlier lesion, involving only a small number of the mitral cells and neurons in the lamina gelatinosa; note the normal appearance of the three outer layers: nerve fibers, glomeruli, and external granular layer. For normal architecture of olfactory bulbs, see also Fig. 1. × 35.

Photographed by Joseph B. Haulenbeek and Louis Schmidt

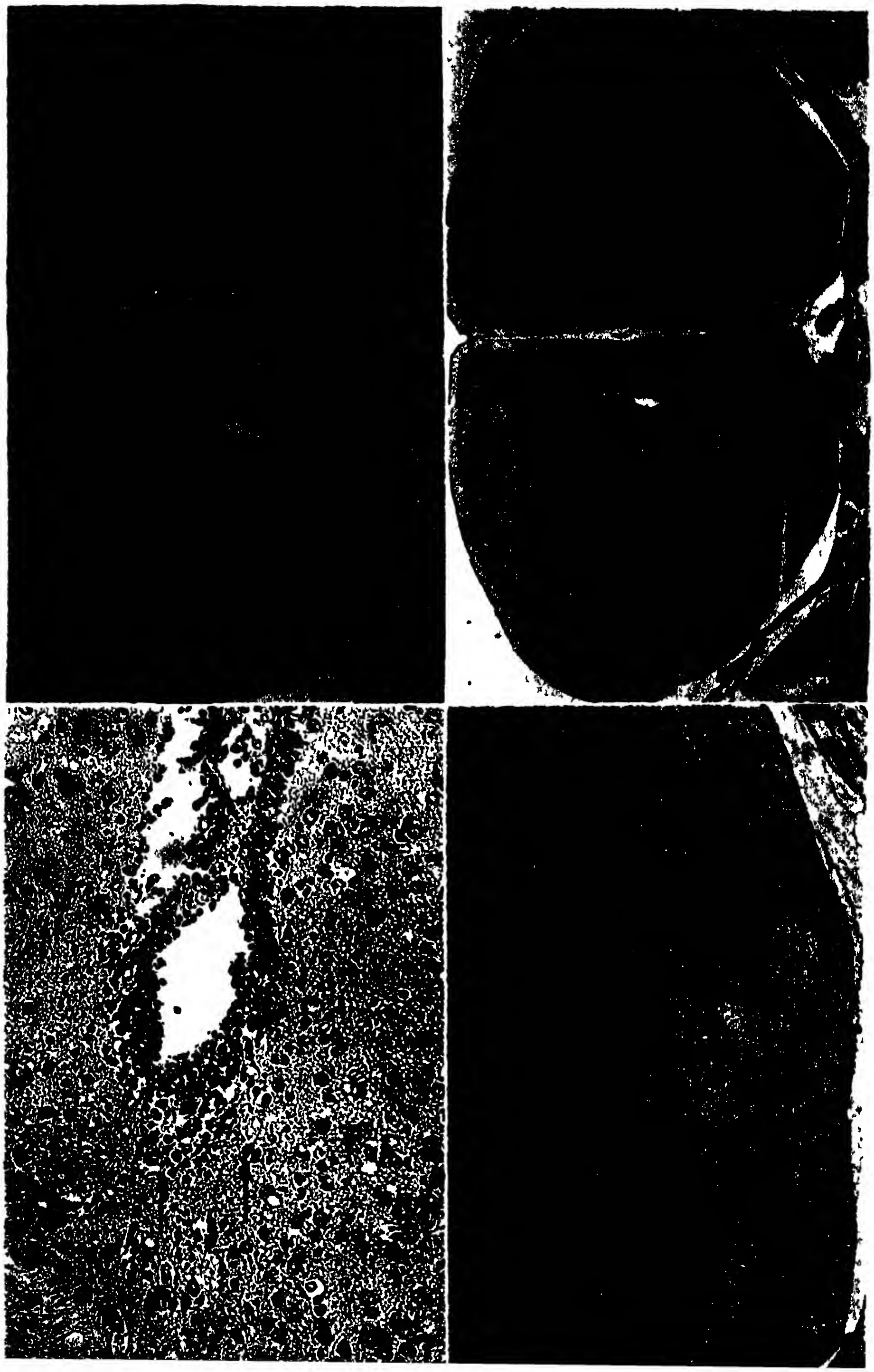
PLATE 8

FIG. 5. Intracerebral inoculation. Arrow points to location of neuronal necrosis around lateral ventricle (see Fig. 7). Letters A and B point to areas to be compared with Fig. 6. × 16.5.

FIG. 6. 4 days after nasal instillation. Compare with Fig. 5 and note necrosis of neurons in the ventromedial aspect of the septum at A and of lateral olfactory gyrus at B. The tuberculum olfactorium (C) presents only a small number of necrotic cells at this level, which are not perceptible at this magnification. × 16.5.

FIG. 7. Periventricular lesion after intracerebral injection. Note necrosis and disorganization of ependyma and shrunken neurons with pycnotic nuclei directly around the ventricle. × 280.

FIG. 8. Higher magnification of necrotic zone in lateral olfactory gyrus shown at B in Fig. 6. × 115.



Photographed by Joseph B. Haulenbeck and Louis Schmidt

(Sabin and Olitsky. Vesicular stomatitis virus. III)

Plate 9

Fig. 9. 4 days after nasal instillation. Arrows point to necrosis in piriform lobe at A, hypothalamus, B, habenular nucleus, C, and cornu Ammonis, D. $\times$ 15.

Fig. 10. Intracerebral inoculation. Arrows point to necrosis of portions of cornu Ammonis and neopallial cortex forming the walls of the lateral ventricles. $\times$ 15.

Photographed by Joseph B. Haulenbeck and Louis Schmidt

(Sabin and Olitsky: Vesicular stomatitis virus. III)

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INFLUENCE OF HOST FACTORS ON NEUROINVASIVENESS OF VESICULAR STOMATITIS VIRUS

IV. VARIATIONS IN NEUROINVASIVENESS IN DIFFERENT SPECIES*

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(Received for publication, October 16, 1937)

The preceding investigations have thrown some light on the cause of variations in the capacity of vesicular stomatitis virus to give rise to encephalitis or myelitis in young and old animals of the same species (mice) (1). This virus, like a number of others, displays also distinct variations in its ability to cause disease of the central nervous system (CNS) in animals of different species under conditions of spontaneous infection or experimental peripheral inoculation. In nature it produces vesicular lesions in horses and cattle but is not known to involve the CNS. In guinea pigs suitable peripheral inoculation also gives rise to vesicular lesions unaccompanied by CNS disease, while in mice of proper age such injections are almost always followed by encephalitis or myelitis, depending upon the site of peripheral inoculation. The present investigation was undertaken to determine, for a single virus, the host factors which are responsible for the protection of the CNS from manifest disease in one species under conditions in which it is constantly involved in animals of another species.

Methods

The technique employed in tracing the progression of the virus in the nervous system and other parts of the body from various sites of inoculation was essentially the same as those already described in the similar studies on mice (1). Necessary variations will be described in the text. The guinea pigs used in the present inves-

* Presented before the American Association of Immunologists and the American Association of Pathologists and Bacteriologists, April 9, 1936, (*Am. J. Path.*, 1936, **12**, 738.)

tigation were of mixed, unknown breed, and the exact ages were known only for the younger animals. Both the New Jersey and Indiana mouse passage strains were employed and tests for virus in guinea pig tissues were made by the intracerebral injection of suitable suspensions in 21 to 30 day old Rockefeller Institute albino mice.

EXPERIMENTAL

Comparative Infectivity of the Virus for Mice and Young and Old Guinea Pigs by the Intracerebral Route.—It is known that guinea pigs develop encephalomyelitis after intracerebral injection of vesicular stomatitis virus (2), but it has not been ascertained whether the minimal amount required to induce manifest CNS disease in them is greater or smaller than that in mice. The purpose of the following experiments, therefore, was to determine in simultaneous tests the comparative susceptibility of the CNS of mice and guinea pigs when the virus was introduced directly into the brain. This information was necessary to indicate whether the absence of manifest CNS disease after peripheral inoculation in guinea pigs was the result of a lesser susceptibility of nervous tissue in general, or of factors related to the mode of virus progression from peripheral sites.

Tests were performed with the Indiana (Ind.) and New Jersey (N. J.) strains, using fresh centrifuged mouse brain suspensions in dilutions from 10^{-1} to 10^{-7} for intracerebral injection into 3 week old mice, young guinea pigs, 8 to 12 days old, and adult guinea pigs 2 months or more of age. The volume injected was 0.03 cc. in each mouse and 0.15 cc. for each guinea pig. One of the difficulties encountered in these tests was due to the fact that broth,[1] which is used as routine for preparing and diluting the virus suspensions, proved to be highly toxic upon intracerebral injection in guinea pigs, 2 months of age or older, more than 50 per cent of the animals dying within less than 12 hours; young and old mice and the young guinea pigs showed no such effect. Although with physiological salt solution as the suspending medium the titer of the virus is almost always tenfold less than with broth, it, nevertheless, had to be used in the comparative intracerebral tests. 110 guinea pigs and forty-eight mice were required to obtain the data necessary for establishing the relative susceptibilities of the CNS of the two species.

The results of three series of tests for each strain of the virus are shown in Table I. The most significant fact for the present investi-

[1] This broth is the kind usually called hormone broth containing chiefly beef heart infusion and 1 per cent peptone.

gation is that the CNS of young guinea pigs and young mice proved to be equally susceptible to both the N. J. and Ind. strains when the virus was injected directly into the brain, *i.e.* the minimal cerebral lethal dose (M.C.L.D.) for mice also caused a clinically apparent CNS disease in young guinea pigs. At the same time two other facts emerge: (*a*) while young and old mice are equally susceptible to intracerebral injection, old guinea pigs (2 months or older) seem to require on an average about 10 times as much virus as young ones (8 to 12 days) for the production of manifest CNS disease; and (*b*) primary flaccid paralysis of the posterior extremities occurred in twenty-five of twenty-seven guinea pigs which succumbed with the Indiana strain and in only two of thirty-three with the N. J. strain. Only about half the number of guinea pigs paralyzed with the Indiana strain died and the others recovered either completely or more often with marked residual paralysis. Guinea pigs succumbing with the N. J. strain exhibited varied encephalitic signs and all died. With respect to the development of flaccid paralysis of the extremities as the primary and chief nervous sign following intracerebral inoculation, the Indiana strain in guinea pigs thus closely resembles poliomyelitis in *rhesus* monkeys.

Comparative Infectivity of the Virus for Young Mice and Young and Old Guinea Pigs by the Nasal Route.—Since there was no record of the effect of nasal instillation of vesicular stomatitis virus in guinea pigs, the present experiment was undertaken to determine it simultaneously in young mice and young and old guinea pigs with a virus suspension whose infectivity by intracerebral inoculation would be established at the same time.

The tests recorded in Table II were carried out simultaneously with those described under Experiment A in Table I, using the same suspensions of virus in broth. 0.075 cc. was instilled in each nostril, using a number of guinea pigs for each of the 10^{-1}, 10^{-2}, and 10^{-3} dilutions. The largest number of guinea pigs, 12 (7 to 9 days old) and 12 (approximately 90 days old), were given the 10^{-2} dilution of the N. J. virus. 15 day old mice were used for control and each received 0.03 cc. of the indicated dilutions.

As was to be expected, all the mice developed encephalitis and died. None of the twelve young and old guinea pigs instilled with the Indiana virus, however, nor any of the fourteen adult guinea pigs

TABLE I

Comparative Infectivity of Vesicular Stomatitis Viruses for Mice and Guinea Pigs by the Intracerebral Route

Strain of vesicular stomatitis virus	Experiment	Animals used	Age	Dilution of virus						
				10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}
Indiana	A Virus in broth	Guinea pigs	8–12 days	n.t.	n.t.	PP4S* PP4D12†	PP5S PP5S	PP8S PP5D21	PP6S PP7S	n.t.
			Over 2 mos.	"	"	D+ 0	D+ 0	D+ D+	0	"
		Mice	21 days	"	"	n.t.	n.t.	3, 3, 4	4, 4, 0‡	"
	B Virus in saline	Guinea pigs	10 days	n.t.	n.t.	n.t.	PP5S 0	E18D22 0	0 0	0 0
			About 10 wks.	E9D10 PP5S PP5R10§ 0	PP3D5 PP5D10 PP5D10 PP5D28	PP6D8 PP6S PP7R11 0	PP6S PP6D11 D6 0	0 0	n.t.	n.t.
		Mice	21 days	n.t.	n.t.	n.t.	n.t.	4, 4, 0	0, 0, 0	0, 0, 0
	C Virus in saline	Guinea pigs	10 days	n.t.	n.t.	n.t.	PP5S 0	0 0	0 0	n.t.
			About 3 mos.	"	"	PP3D5 PP7D16	PP5D7 0	0 0	n.t.	"
		Mice	21 days	"	"	n.t.	n.t.	4, 5, 0	0, 0, 0	0, 0, 0

New Jersey	A Virus in broth	Guinea pigs	10 days	n.t.	n.t.	n.t.	E4D6‖ E6D9	D+ E6D11	D4 E6D8	n.t.
			About 3 mos.	D+ D+	E3D4 E4D6	D+ PP3D4	D+ PP5D8	D+ 0	0 0	"
		Mice	21 days	n.t.	n.t.	n.t.	n.t.	3, 3, 4	3, 4, 5	"
	B Virus in saline	Guinea pigs	10 days	n.t.	n.t.	n.t.	D2 E3D3	E3D8 E7D9	D4 E4D7	E8D13 0
			About 3 mos.	"	E3D4 E3D5 E4D6	E3D5 E3D5 E4D5 E7D11	E4D6 E6D10 E7D10 E11D13	E7D10 E7D10	D7 0	n.t.
		Mice	21 days	"	n.t.	n.t.	n.t.	3, 4, 4	3, 3, 0	0, 0, 0
	C Virus in saline	Guinea pigs	10 days	n.t.	n.t.	n.t.	n.t.	E4D5 E4D7	0 0	0 0
			Over 3 mos.	"	"	"	E4D7 0	0 0	0 0	n.t.
		Mice	21 days	"	"	"	n.t.	4, 5, 0	0, 0, 0	0, 0, 0

0 = inoculated animal remained well.
n.t. = not tested.
D+ = died within less than 12 hours: broth toxicity.
* PP4S = flaccid paralysis of posterior extremities 4 days after inoculation; survived.
† PP4D12 = flaccid paralysis of posterior extremities 4 days after inoculation; died 12th day.
‡ 4, 4, 0 = three mice inoculated of which two died on the 4th day and one survived.
§ PP5R10 = flaccid paralysis of posterior extremities 5 days after inoculation; complete recovery on 10th day.
‖E4D6 = signs of encephalitis 4th day and died 6th day.

which received the N. J. strain showed any signs of disease. One of the twelve 7 to 9 day old guinea pigs instilled with the 1:100 dilution of N J. virus developed distinct encephalitic signs (coarse tremors, incoordination, etc.) on the 5th day which lasted for only 3 to 4 days, *the animal making a complete recovery. Further experience with nasal instillation of the N. J. strain indicates that guinea pigs over* 2 months of age show no signs of disease whatever, while no more and probably less than one of about twenty of the very young ones exhibits some transitory encephalitic signs. When the results for the

TABLE II

Comparative Infectivity of Vesicular Stomatitis Viruses for Guinea Pigs and Mice by the Nasal Route

Strain of virus	Animals used	Age	Dilution of virus		
			1:10	1:100	1:1000
Indiana	Guinea pigs	8–12 days	0, 0	0, 0	0, 0
		Over 2 mos.	0, 0	0, 0	0, 0
	Mice	15 days	4, 4, 4	4, 5, 5	5, 6, 6
New Jersey	Guinea pigs	7–9 days	0, E?D6	0, 0, 0, 0, 0, 0 0, 0, 0, 0, 0, E5 Rec.	n.t.
		About 3 mos.	0, 0	0, 0, 0, 0, 0, 0 0, 0, 0, 0, 0, 0	n.t.
	Mice	15 days	4, 4, 4	4, 4, 5	n.t.

E?D6 = dubious signs of encephalitis; died 6th day.
E5 Rec. = signs of encephalitis appeared on 5th day; complete recovery.
Other legends as in Table I.

young animals of the two species are compared it appears that while both are equally susceptible to intracerebral inoculation, nasal instillation of the virus (in adequate amounts) constantly leads to encephalitis in young mice and, with only rare exceptions, not in the young guinea pigs. This in itself suggested that the absence of manifest CNS disease after peripheral inoculation in guinea pigs was not the result of a lower susceptibility of the entire nervous system, but was influenced rather by factors which modified the progression of nasally instilled virus in this species.

Spread of the Virus (N. J. Strain) into Central Nervous Systems of Young and Old Guinea Pigs after Nasal Instillation.—It will be recalled that in old mice, remaining entirely well after nasal instillation of the virus, the CNS was, nevertheless, invaded along the olfactory pathway and that the progression of the virus appeared to be arrested somewhere in the anterior rhinencephalon (1). The object of the following experiment was to disclose whether or not the resistance of guinea pigs was brought about by a similar mechanism.

TABLE III

Spread of Vesicular Stomatitis Virus (N. J. Strain) into Central Nervous System of Young and Adult Guinea Pigs after Nasal Instillation

Experiment	Time after nasal instillation of virus 0.5 × 10⁵ M.C.L.D.	Presence of virus in					
		Young (10–12 days)			Adult (10–12 wks.)		
		Blood	Anterior rhinencephalon	Rest of brain	Blood	Anterior rhinencephalon	Rest of brain
	days						
A	2	0, 0, 0	3, 3, 0*	0, 0, 0	0, 0, 0	0, 0, 0	0, 0, 0
	4	0, 0, 0	3, 6, 0	3, 3, 3	0, 0, 0	0, 0, 0	0, 0, 0
	8	0, 0, 0	4, 0, 0	0, 0, 0	0, 0, 0	0, 0, 0	0, 0, 0
B	2	0, 0, 0	2, 3, 4	0, 0, 0	0, 0, 0	0, 0, 0	0, 0, 0
	4	0, 0, 0	2, 2, 6	3, 0, 0	0, 0, 0	0, 0, 0	0, 0, 0
	8	0, 0, 0	3, 3, 3	3, 3, 3	0, 0, 0	0, 0, 0	0, 0, 0
C	6	n.t.	n.t.	n.t.	0, 0, 0	0, 0, 0	0, 0, 0
	10	"	"	"	0, 0, 0	0, 0, 0	0, 0, 0

* 3, 3, 0 = material injected intracerebrally in three mice of which two died on the 3rd day and one remained well.

Groups of guinea pigs, 10 to 12 days, and 10 to 12 weeks of age, were given nasal instillations of approximately 50,000 M.C.L.D. of the N. J. virus. At the intervals indicated in Table III, different animals were sacrificed and their blood and parts of the brain were tested for virus by intracerebral inoculation in mice. For this series of tests the brain was divided, as in mice, into two parts: the olfactory bulbs and structures ventral to the rhinal fissure up to the optic chiasm constituted one part, referred to as the anterior rhinencephalon, and a number of pieces from representative regions of the rest of the brain constituted the other. The former was ground up in 3 cc. of broth and the latter in 6 cc. of which 0.03 cc. was injected into each of three mice. Great care was taken to establish (by

examination of film preparation, culture, and passage) that mice which succumbed did so as a result of infection with vesicular stomatitis virus; it was in the course of such tests that toxoplasma were isolated on one occasion (3).

The results shown in Table III present a different picture from that obtained in mice. In the adult guinea pigs no virus was detected at any time between the 2nd and 10th days in either the blood or any part of the brain. In the young guinea pigs, on the other hand, virus was regularly found in the brain with none in the blood. In the animals sacrificed on the 2nd day virus was detected only in the anterior rhinencephalon, but in those killed on the 4th and 8th days after nasal instillation it was present there and also in the remainder of the brain. While the fact that virus was detectable only in the anterior rhinencephalon on the 2nd day is to some extent evidence against the widespread dissemination expected of spread in an "open system," it was, nevertheless, clear that it subsequently involved other parts of the brain and was not arrested in the same manner or site as in the resistant mice. It was, therefore, necessary to determine whether this later spread of the virus was diffuse and without relation to the tract connections of the olfactory pathway, (in which case it would be difficult to understand the absence of clinical CNS disease, manifested so constantly in response to the intracerebral injection of the minutest amounts of virus) or whether the progression might still be limited to definite areas, the arrest however, occurring somewhat more posteriorly than in mice, but still in clinically "silent" zones.

This premise was tested in an experiment, recorded in Table IV, in which the rest of the brain was subdivided into several portions. After cutting away the anterior rhinencephalon, the cortex (no separation was then made between the neopallial and olfactory portions) was peeled away from the brain stem and portions of the parietal and occipital regions (including, of course, the piriform lobes and cornu Ammonis) were saved for tests. The diencephalon (including the pars optica hypothalami) and mesencephalon, the pons and medulla, and the cerebellum were the other regions examined for virus. 10 day old guinea pigs, which were given about 500,000 M.C.L.D. of the N. J. virus, were sacrificed at 18 hours, 3, 7, and 10 days after nasal instillation. The blood at all these intervals, and the brain tissue of those killed at 18 hours and 3 days showed no virus. In the guinea pig sacrificed on the 7th day abundant virus was demonstrated in the anterior rhinencephalon, the cortical regions, and the diencephalon and mesencephalon, while none was found in the pons and medulla and cerebellum. On

the 10th day a small amount of virus was still detectable in the anterior rhinencephalon, and diencephalon and mesencephalon, but none in any of the other regions.

These results seemed to indicate that the virus did not spread indiscriminately throughout the CNS, and another series of experiments were undertaken to localize more definitely the affected zones and the site of apparent arrest of progression.

Fate of Nasally Instilled Virus and Site of Arrest in CNS.—In the following experiment an attempt was made to ascertain (*a*) the fate

TABLE IV

Distribution of Nasally Instilled Vesicular Stomatitis Virus in the Central Nervous System of Young Guinea Pigs

Time after nasal instillation of virus 0.5 × 10⁶ M.C.L.D.	Presence of virus in						
	Blood	Anterior rhinencephalon	Parietal cortex	Occipital cortex	Diencephalon and mesencephalon (+ pars optica hypothalami)	Cerebellum	Pons and medulla
18 hrs.	0, 0, 0	0, 0, 0	0, 0, 0	0, 0, 0	0, 0, 0	0, 0, 0	0, 0, 0
3 days	0, 0, 0	0, 0, 0	0, 0, 0	0, 0, 0	0, 0, 0	0, 0, 0	0, 0, 0
7 days*	0, 0, 0	3, 5, 0	2, 3, 4	3, 3, 4	3, 3, 3	0, 0, 0	0, 0, 0
10 days†	0, 0, 0	6, 0, 0	0, 0, 0	0, 0, 0	5, 6, 0	0, 0, 0	0, 0, 0

* The guinea pig sacrificed on the 7th day had fever on the 6th (104.8°F.) and 7th (105.2°F.) days.

† The guinea pig sacrificed on the 10th day had fever on the 6th (104.6°F.) and seventh (104.5°F.) days.

Other legends as in preceding tables.

of the virus in the nasal mucosa especially in relation to its subsequent invasion of the CNS, and (*b*) whether or not its localization in the brain was in accord with a progression along the central connections of the olfactory neurons.

The age of the animal and the dose of virus were the controlled variables. The young guinea pigs were 7 to 10 days old and the old ones were at least 6 months of age. One group of each age received a nasal instillation of about 500,000 M.C.L.D. of the N. J. virus and another about 50,000 M.C.L.D. The nasal mucosa and various parts of the brain were tested for virus at 3 hours, 2, 5, and 7 days. The entire nasal mucosa was ground with alundum and 5 cc. of broth; after horizontal centrifugation of the suspension at about 2000 R.P.M. for 45 to 60 minutes,

the supernatant liquid and dilutions prepared from it, were injected intracerebrally in mice. The anterior rhinencephalon was obtained in the usual manner, but the remainder of the brain was dissected somewhat differently. Following along the very sharp and distinct rhinal fissure the cortex was easily separated into the neopallial portion (non-olfactory), and the archi- and paleo-pallial portions containing the piriform lobes, and cornu Ammonis (olfactory zones). The diencephalon (including the pars optica hypothalami) and mesencephalon were tested individually while the pons, medulla, and cerebellum were pooled.

The results shown in Table V may be summarized as follows: The *young guinea pigs* receiving *500,000* M.C.L.D. of virus exhibited: *3 hours later*, virus only in the undiluted suspension of nasal mucosa (*i.e.* about 100–200 M.C.L.D.) and none in any tested part of the brain; *2 days later*, only a trace of virus in the nasal mucosa but a considerable amount in the anterior rhinencephalon with none in any of the other regions of the brain; *5 days later*, small amount of virus in anterior rhinencephalon and none detectable in nasal mucosa or other regions of the brain; *7 days later*, an appreciable amount in the anterior rhinencephalon, and piriform and hippocampal regions with none found elsewhere in the brain or nasal mucosa.

The *young ones* instilled with *50,000* M.C.L.D. of virus exhibited: *3 hours later*, none in the nasal mucosa or brain; *2 days later*, abundant virus in the mucosa (present in the 10^{-2} but not in the 10^{-3} dilution) as well as in the anterior rhinencephalon; *5 days later* virus absent, or in smaller amount, in nasal mucosa, but present in considerable quantity in anterior rhinencephalon, piriform and hippocampal regions, and some in the diencephalon but not elsewhere in the brain; *7 days later*, none found in the nasal mucosa or any part of the brain with the exception of the diencephalon which contains a considerable amount.

The *old guinea pigs* given *500,000* M.C.L.D. of virus exhibited: *3 hours later*, none in nasal mucosa or brain; *2 days later*, abundant virus in nasal mucosa (10^{-2} dilution positive) and a trace in the anterior rhinencephalon, with none in the other regions of the brain; *5 and 7 days later*, a trace of virus in the nasal mucosa with none in any part of the brain.

The *old guinea pigs* given *50,000* M.C.L.D. of virus exhibited: *3 hours later*, none in nasal mucosa or brain; *2 days later*, none or undetermined small amount in nasal mucosa and none in brain; *5 days later*, trace in nasal mucosa and anterior rhinencephalon with none elsewhere in brain; *7 days later*, none in nasal mucosa or brain.

It appears, therefore, that as in mice (1), almost all the virus instilled into the nose disappears within a very short time, to such an extent that in three of the four guinea pigs tested within 3 hours none was detectable, indicating that of the 50,000 or 500,000 M.C.L.D. of instilled virus less than 100 M.C.L.D. remained fixed or in an infective state. It also seems clear that in both the young and the old guinea pigs there may be quite an appreciable increase in the amount of virus

in the nasal mucosa within the first 2 days which, however, disappears rapidly thereafter. When the results of the present experiment are

TABLE V

Fate of Nasally Instilled Vesicular Stomatitis Virus (N. J.) and Site of Arrest in Central Nervous System as Influenced by Amount of Virus and Age of Guinea Pigs

Age of guinea pigs	Amount of virus instilled	Time after nasal instillation	Guinea pig No.*	Presence of virus in: Nasal mucosa in 5 cc.: Undiluted	1:10	1:100	1:1000	Anterior rhinencephalon	Piriform and hippocampus	Diencephalon + pars optica hypothalami	Mesencephalon	Pons, medulla, and cerebellum	Neopallium
7–10	500,000	3 hrs.	1	3, 7†	0, 0	n.t.	n.t.	0, 0	n.t.	0, 0	0, 0	0, 0	n.t.
days	M.C.L.D.	2 days	2	4, 0	0, 0	0, 0	0, 0	3, 3	0, 0	0, 0	0, 0	0, 0	"
		5 "	3	0, 0	0, 0	0, 0	0, 0	3, 0	0, 0	0, 0	0, 0	0, 0	0, 0
		7 "	4	0, 0	0, 0	0, 0	n.t.	3, 3	3, 4	0, 0	0, 0	0, 0	0, 0
	50,000	3 hrs.	5	0, 0	0, 0	n.t.	n.t.	0, 0	n.t.	0, 0	0, 0	0, 0	n.t.
	M.C.L.D.	2 days	6	2, 2	2, 2	2, 3	0, 0	2, 2	—‡	—	—	—	—
		5 "	7	—	0, 0	0, 0	0, 0	2, 3	2, 3	2, 0	0, 0	0, 0	0, 0
		7 "	8	0, 0	0, 0	0, 0	n.t.	0, 0	0, 0	2, 2	0, 0	0, 0	0, 0
Over 6	500,000	3 hrs.	9	0, 0	0, 0	n.t.	n.t.	0, 0	n.t.	0, 0	0, 0	0, 0	n.t.
mos.	M.C.L.D.	2 days	10	2, 2	2, 2	2, 3	"	3, 0	"	0, 0	0, 0	0, 0	"
		5 "	11	5, 0	0, 0	0, 0	"	0, 0	"	0, 0	0, 0	0, 0	"
		7 "	12	(4), 0	0, 0	0, 0	"	0, 0	"	0, 0	0, 0	0, 0	"
	50,000	3 hrs.	13	0, 0	0, 0	n.t.	"	0, 0	"	0, 0	0, 0	0, 0	"
	M.C.L.D.	2 days	14	—	0, 0	0, 0	"	0, 0	"	0, 0	0, 0	0, 0	"
		5 "	15	0, 0	6, 6	0, 0	"	2, 0	"	0, 0	0, 0	0, 0	"
		7 "	16	0, 0	0, 0	0, 0	"	0, 0	"	0, 0	0, 0	0, 0	"

* None of the guinea pigs sacrificed for these tests exhibited any signs of disease.

† The brain of at least one sick or dead mouse in each group of two was cultured and subinoculated in animals in order to establish that the illness and death were due to virus.

‡ Dashes indicate that material was contaminated with pathogenic bacteria.

(4) = mouse died on 4th day but material was unsuitable for establishing cause of death.

combined with those of Table III, it is to be noted that among 16 old guinea pigs, a trace of virus was found only in the brains of two (lim-

ited to the anterior rhinencephalon), indicating that in spite of the multiplication of virus which may occur in the nasal mucosa it cannot as a rule invade the brain and that when it does get into the brain on occasions, it can apparently neither multiply nor progress beyond the anterior olfactory region. In the young guinea pigs, however, there is evidence of constant invasion of the brain, the virus becoming detectable in the anterior rhinencephalon on the 2nd day at which time the other regions of the brain show none. It then spreads posteriorly apparently in accord with a definite order localizing in the piriform and hippocampal regions but not in the neopallial regions of the cortex, and occasionally in the diencephalon (or only the pars optica hypothalami) but apparently not beyond. The finding of virus only in the diencephalon in the brain of one guinea pig on the 7th day merely suggests the possibility that it may disappear last from the areas which are last to be involved. This type of localization, however, is completely in accord with progression of the virus within the neurons of the olfactory pathway and not at all in agreement with a spread of the virus in an open system. It can also be inferred, therefore, that the absence of apparent CNS disease in nasally instilled guinea pigs may be effected by the same mechanism which determined the resistance of old mice. The difference between the two species is in (*a*) the sites at which the progression of nasally instilled virus is arrested, and (*b*) in the fact that 7 to 10 day old guinea pigs appear to possess "barriers" which are acquired by mice only at a much later age.

Spread of Virus after Intramuscular or Pad Inoculation.—Although in past investigations by others, large numbers of guinea pigs have been injected into the pads with the vesicular contents from horses and cattle, or with the pad passage or more recent brain passage strains, there is no record of any paralysis or other signs of CNS disease occurring in these animals. When the present investigation was begun, twelve guinea pigs, approximately 1 month old, were injected intracutaneously and subcutaneously in the pad of one posterior extremity with mouse brain virus and one developed typical flaccid paralysis of the inoculated posterior extremity on the 5th day followed by paralysis of the opposite leg, and ascending paralysis resulting in death on the 9th day after inoculation. It is regrettable

that no search for virus was made, although no other cause for these signs was found. In view of this unexpected finding many other guinea pigs, and particularly very young ones, were inoculated in the same manner without our ever again observing any evidence of CNS disease.

TABLE VI

Spread of Vesicular Stomatitis Virus (N. J.) after Intramuscular or Pad Inoculation

Site of inoculation	Age and average weight of guinea pigs	Time after inoculation	Presence of virus in: Blood	Spleen	Regional lymph nodes	Right sciatic nerve	Right sacral and lumbar ganglia	Spinal cord	Left sacral and lumbar ganglia	Left sciatic nerve
		days								
Intracutaneously and subcutaneously in right pad (about 10^6 M.C.L.D.)	About 1 mo.; 300 gm.	3	0, 0, 0	n.t.	n.t.	0, 0, 0	0, 0, 0	0, 0, 0	0, 0, 0	0, 0, 0
		6	0, 0, 0	"	"	0, 0, 0	0, 0, 0	0, 0, 0	0, 0, 0	0, 0, 0
		10	0, 0, 0	"	"	0, 0, 0	0, 0, 0	0, 0, 0	0, 0, 0	0, 0, 0
	About 1 mo.; 300 gm.	6	0, 0, 0	"	"	0, 0, 0	0, 0, 0	0, 0, 0	0, 0, 0	0, 0, 0
		10	0, 0, 0	"	"	0, 0, 0	0, 0, 0	0, 0, 0	0, 0, 0	0, 0, 0
Intramuscularly in right calf muscles (about 10^6 M.C.L.D.)	10 days; 167 gm.	2	0, 0, 0	0, 0, 0	0, 0, 0	0, 0, 0	0, 0, 0	0, 0, 0	0, 0, 0	0, 0, 0
		4	0, 0, 0	0, 0, 0	2, 3, 3	0, 0, 0	0, 0, 0	0, 0, 0	0, 0, 0	0, 0, 0
		8	0, 0, 0	0, 0, 0	0, 0, 0	0, 0, 0	0, 0, 0	0, 0, 0	0, 0, 0	0, 0, 0
	Over 3 mos.; 580 gm.	2	0, 0, 0	0, 0, 0	0, 0, 0	0, 0, 0	0, 0, 0	0, 0, 0	0, 0, 0	0, 0, 0
		4	0, 0, 0	0, 0, 0	0, 0, 0	0, 0, 0	0, 0, 0	0, 0, 0	0, 0, 0	0, 0, 0
		8	0, 0, 0	0, 0, 0	0, 0, 0	0, 0, 0	0, 0, 0	0, 0, 0	0, 0, 0	0, 0, 0

The following tests were undertaken to determine whether or not the virus injected intramuscularly or into the pads, was capable of invading the CNS along the nerves supplying the inoculated sites.

One group of 1 month old guinea pigs was given about 10^6 M.C.L.D. of mouse brain virus (N. J.) intracutaneously and subcutaneously in the right pad. Two animals were sacrificed at 3, 6, and 10 day intervals and the structures, indicated

in Table VI, were pooled and injected intracerebrally in mice. Three other groups of guinea pigs (young and adult) were given approximately the same amount of virus intramuscularly in the right posterior extremity, and various tissues were tested at 2, 4, 6, 8, and 10 day intervals.

The results shown in Table VI indicated that in none of these animals was virus found in the blood, peripheral nerves, spinal ganglia, or spinal cord. Virus was detected but once and that was in the regional lymph nodes of a 10 day old guinea pig, 4 days after intramuscular injection. It was clear, therefore, that in young and old guinea pigs, as in old mice, absence of manifest CNS disease following this form of peripheral inoculation was associated with an inability of the virus to invade the nervous system. In old mice it will be recalled, this was associated with inability of the virus to multiply at the site of inoculation. Although vesicular stomatitis virus is known to produce definite vesicular lesions in the pads of guinea pigs, and, therefore, presumably to be capable of local multiplication, it was nevertheless desirable to establish this fact beyond question particularly for the virus used in these experiments, which has undergone over 100 brain to brain passages in mice.

Twelve 8 to 10 day old guinea pigs and an equal number of old animals weighing on the average 1000 gm. each were given about 10^6 M.C.L.D. of the N. J. virus into the pad of one posterior extremity, part by "tunneling" and the remainder subcutaneously, and a similar volume of plain broth in the other for control. Only the pads inoculated with virus developed lesions; these were distinct on the 3rd day and were generally less marked in the young than in the old. Histological sections of some of the pads revealed the characteristic picture including intranuclear inclusions and necrosis of epithelial cells. Animals of each group were sacrificed at 2, 22, and 72 hours after inoculation. The pads inoculated with virus were washed with alcohol and ether, dissected away, and ground up with alundum and broth. The pad of an old guinea pig, weighing usually 0.3 gm., was ground up with 6 cc. of broth, that of a young one, weighing 0.1 gm., with 5 cc. of broth. The suspensions were centrifuged at about 2000 R.P.M. for 45 to 60 minutes, and the clear supernatant liquids (designated in the old as the 1:20 dilution and for the young as the 1:50) and further dilutions of them were injected intracerebrally in mice.

The results of this experiment (Table VII) leave no doubt as to the local multiplication of the virus. It is clear, therefore, that local multiplication does not in itself determine the capacity of this virus to invade the peripheral nerves.

Effect of Intrasciatic Injection of Virus.—Inoculation of the virus directly into the sciatic nerve of resistant mice resulted in a fatal ascending myelitis in six of eleven animals, indicating that virus progression along peripheral nerve fibers was possible and that the chief barrier to invasion of the CNS was in some structure or structures at the site of intramuscular or pad inoculation (1).

To determine whether or not the same was true for guinea pigs, twelve of them were given a 10 per cent suspension of N. J. virus into the right sciatic nerves. An equal number of guinea pigs which, for control, were injected intramuscularly, in the neighborhood of the same region of the sciatic nerve, remained well.

TABLE VII

Local Multiplication of Vesicular Stomatitis Virus (N. J.) in Pads of Young and Old Guinea Pigs

Age and average weight of guinea pigs	Time after inoculation of about 10^6 M.C.L.D.	Dilution of pad suspension						
		1:20	1:50	1:100	1:1000	1:10,000	1:100,000	1:1,000,000
	hrs.							
8–10 days; 121 gm.	2	n.t.	3, 5	n.t.	0, 0	0, 0	n.t.	n.t.
	22	"	2, 2	"	3, 5	8, 9	"	"
	72	"	4, 5	"	5, 10	0, 0	0, 0	0, 0
Over 3 mos.; 1000 gm.	2	0, 0	n.t.	0, 0	0, 0	0, 0	n.t.	n.t.
	22	2, 3	"	2, 3	3, 4	2, 5	"	"
	72	4, 4	"	0, 0	5, 7	0, 0	0, 0	0, 0

Four of the twelve guinea pigs (Table VIII) receiving the virus in the sciatic nerve, developed typical flaccid paralysis of the posterior extremities, which ascended and caused death in three instances, the fourth animal having been sacrificed for virus tests. Some of the guinea pigs without nervous signs exhibited fever (104–106°F.) but no spread of virus was demonstrable in association with it. Tests for virus in a guinea pig dying with paralysis revealed its presence in the lumbar and cervical portions of the cord, the medulla, and brain, but not in either one of the sciatic nerves. The failure to detect virus in the inoculated sciatic nerve at the time of paralysis and its presence in the lumbar cord, observed also in another guinea pig, duplicates the experience with this virus in old mice and its probable significance has already been discussed (1).

It is clear from these experiments that vesicular stomatitis virus injected directly into a peripheral nerve like the sciatic can invade

TABLE VIII

Effect of Intrasciatic Injection of Virus

Age and average weight	Guinea pig No.	Result	Presence of virus in: Right sciatic nerve	Left sciatic nerve	Lumbar cord	Cervical cord	Medulla	Brain	Spleen
10–12 days; 200 gm.	1	Fever 5th and 6th days; paralysis of posterior extremities 5th day; dead 7th day	0, 0	0, 0	2, 2	3, 3	3, 3	2, 2	n.t.
	2	Fever* 6th, 7th, 8th, 10th days; remained well							
10 weeks; 500 gm.	3	Fever 5th, 7th, 10th days; paralysis of posterior extremities 5th day; encephalitic signs, 13th day; dead 15th day							
	4	Fever 10th, 11th, 12th days; remained well							
About 6 mos.; 600 gm.	5	Fever 7th, 10th, 11th, 12th days; remained well							
	6	Fever 11th, 12th, 13th days (105–106°F.); no nervous signs; sacrificed 13th day	0, 0	0, 0	0, 0	0, 0	0, 0	0, 0	0, 0
6–10 wks.; 350–500 gm.	7	No fever; remained well							
	8	" " " "							
	9	Fever 3rd, 4th, 6th days; paralysis right posterior extremity 5th day; paralysis both posterior extremities 6th day; dead 7th day							
	10	No fever; paralysis of both posterior extremities 6th day; no change 7th day; sacrificed 7th day	0, 0, 0	0, 0, 0	3, 5, 0	0, 0, 0	0, 0, 0	0, 0, 0	0, 0, 0
	11	No fever; remained well							
	12	Remained well							

* Fever refers to a distinct rise in temperature of 1.5°F. or more over the preceding level and reaching at least 104°F.

the CNS of guinea pigs which it is incapable of accomplishing after inoculation into a site supplied by this nerve. In view of the fact also that the CNS is highly susceptible and that the virus can multiply in the area of peripheral injection affecting tissue cells supplied by these peripheral nerves, one is forced to consider, by a process of elimination, that the terminal, specialized nerve endings may constitute the real barrier to invasion of the CNS.

DISCUSSION

A number of observations made in the present investigation indicate that a neurotropic virus to which the CNS of two species is equally susceptible may, after peripheral inoculation, cause encephalitis or myelitis in one but not in the other, the difference being determined by variations in the character of the nervous pathways along which the virus must spread. When vesicular stomatitis virus is introduced directly into the brain, the minimal dose (1 M.C.L.D.) can produce encephalitis in young guinea pigs as readily as in young mice. On the other hand, while 100 to 1000 M.C.L.D. instilled intranasally in young mice, invariably gives rise to a fatal encephalitis, 1000 times that amount is, with very rare exceptions, entirely unassociated with any clinical signs of CNS involvement. Since the nasally instilled virus, nevertheless, regularly invades the CNS of young guinea pigs and spreads through the olfactory regions of the brain along pathways which it has been shown to utilize in susceptible mice, it would appear that some variation in these nervous pathways is responsible for the arrest of its progression in the terminal olfactory areas or diencephalon. After inoculation into tissues supplied by spinal nerves (*e.g.* sciatic) the virus undergoes local multiplication in young guinea pigs as in young mice, but causes myelitis only in the latter while in the former it fails to invade the nervous tissue altogether. The fact that direct intrasciatic injection is frequently followed by a fatal ascending myelitis tends to eliminate the peripheral nerves themselves as the barriers to invasion of the CNS and forces consideration of a variation in structures, such as the myoneural junctions or other specialized nerve endings, through which is effected the intimate relationship between the axis cylinders and the inoculated tissues. The rôle of localized barriers in hosts of different age or species in preventing or

TABLE IX

Localized Barriers as a Factor in Preventing or Arresting Infection of the Central Nervous System (Vesicular Stomatitis Virus)

Host	Route of inoculation	Susceptibility of young and old	Probable site of barrier
Mouse	Intracerebral	Both equally susceptible	0
	Intramuscular Subcutaneous, pad	Young, regularly myeloencephalitis Old, 100 per cent resistant	Muscle or myoneural junction; epithelium or specialized nerve endings
	Intraocular	Young, regularly encephalitis Old, more than 90 per cent resistant	Retina
	Intranasal	Young, regularly encephalitis Old, resistance varies from 50 to 90 per cent	In CNS, anterior olfactory region (between 2nd and 3rd olfactory neurons?)
Guinea pig	Intracerebral	Young guinea pigs and mice equally susceptible, old guinea pigs somewhat less	0
	Intramuscular Subcutaneous Intracutaneous	Young and old resistant	Myoneural junction and specialized nerve endings
	Intranasal	Young and old resistant	Young, (*a*) between olfactory cortex and remainder of brain (*b*) between diencephalon (or pars optica hypothalami) and remainder of brain Old, generally between nasal mucosa and CNS

arresting infection of the CNS with vesicular stomatitis virus is summarized in Table IX.

There are many other instances in nature where a virus is highly neuroinvasive in one species and apparently not at all (or very rarely) in another. As a classical example one may cite the virus which, while causing only herpes simplex in human beings, will, when transferred to the skin, cornea, or mucous membrane of the rabbit, cause not only local lesions like those in man, but also clinically apparent and fatal disease of the CNS. Pseudorabies (4) and B virus (5, 6) which are pantropic and highly neuroinvasive in the rabbit, will cause encephalitis when injected intracerebrally but not peripherally (skin, muscle) in the *rhesus* monkey; this is associated with a loss of the capacity to produce lesions in non-nervous tissue of the monkey in the case of pseudorabies but not of B virus. That variation in the character of the specialized nerve endings in different species may be involved here, as in the case of vesicular stomatitis virus, receives some support in Hurst's finding that intrasciatic injection of pseudorabies virus in the *rhesus* monkey frequently causes myeloencephalitis (4).

The observations made in this series of studies on vesicular stomatitis virus in different hosts may supply a pattern for at least one type of inapparent infection of the CNS. It is generally assumed, for example, that the majority of the human population are subject to inapparent infection with the virus of poliomyelitis, while only rare individuals exhibit the clinically apparent form of the disease. Faber (7) has thus postulated on theoretical grounds that poliomyelitis virus in man, spreading axonally along the olfactory pathways, might be halted in its progression in silent zones of the CNS in the majority of instances. That such a thing is possible and actually occurs with another neurotropic virus is evident from the demonstrated behavior of vesicular stomatitis in guinea pigs which seems to offer a remarkable parallel for many of the manifestations of poliomyelitis in man. There are the rare, individual guinea pigs which after nasal instillation exhibit clinical signs of CNS disease, while in the majority which appear well there is, nevertheless, transitory multiplication of the virus in the nasal mucosa with involvement of definitely limited zones in the brain; and to accentuate the parallel

even further it may be stated here that antiviral bodies regularly appear in the blood of all guinea pigs regardless of the extent of CNS involvement. (Detailed studies on the immune response to arrested infection with vesicular stomatitis virus will be presented in a future communication.) Other patterns of inapparent infection of the CNS, which have been described recently, must also be considered. Burnet (8), for example, showed that louping ill virus which regularly causes encephalitis in the mouse, is followed by no signs of disease when the virus is injected by intracerebral or peripheral routes in young or old rats. After nasal instillation in rats there appears, nevertheless, to *be local multiplication of the virus and an invasion of the brain that* is generally limited to the olfactory bulbs. The observations of Webster and Clow (9) on mice with a high inborn resistance to the virus of St. Louis encephalitis demonstrate still another type of clinically inapparent infection of the CNS; intracerebral and peripheral inoculation are equally harmless in these mice, and nasal instillation is followed by as widespread an invasion of the brain as in the susceptible animals. The absence of nervous signs here is correlated with a distinctly lower level of virus multiplication throughout the CNS, rather than with localized barriers to virus progression.

SUMMARY AND CONCLUSIONS

Peripheral inoculation of vesicular stomatitis virus is constantly followed by myelitis or encephalitis in young mice, but not in young (or old) guinea pigs. The cause of this variation was elucidated by investigating the fate of the virus after inoculation by a number of different routes.

Direct intracerebral injection of minimally infective amounts of virus was found to be equally fatal for young mice and young guinea pigs, indicating that the central nervous system as a whole was as easily injured by the virus in one species as in the other.

The events following nasal instillation of the virus varied in young and old guinea pigs. While there appeared to be a transitory multiplication of virus in the nasal mucosa of both young and old, the central nervous system was regularly invaded only in the young. In these, virus was first found only in the anterior rhinencephalon; later it spread to the piriform and hippocampal (olfactory regions)

but not to the neopallial portions of the cortex, and the only other area to exhibit virus was the diencephalon (including the pars optica hypothalami), where its further progression was apparently arrested.

Absence of central nervous system disease following inoculation into sites supplied by spinal nerves (*e.g.* sciatic) was found to be due to inability of the virus to invade the nerves.

Since direct intrasciatic inoculation frequently led to a fatal ascending myelitis, it was evident that the central nervous system could be invaded along the spinal nerves, and that they did not constitute the main barrier. Furthermore, since multiplication of virus was demonstrated in tissues supplied by the spinal nerves, a process of elimination made it seem possible that the specialized, terminal nerve endings might be the structures which prevent the progression of the virus from the infected tissues to the axons and hence also to the central nervous system.

7 day old guinea pigs (or guinea pigs as a species) were thus found to possess much the same type of barriers to the progression of peripherally inoculated vesicular stomatitis virus as are acquired by mice at a considerably later age.

In a discussion of the present data, they have been correlated with known variations in neuroinvasiveness of other viruses and their bearing on the nature of inapparent or subclinical infections of the central nervous system has been considered.

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FURTHER OBSERVATIONS ON INTRANUCLEAR INCLUSIONS PRODUCED BY NON-VIRUS MATERIALS

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The purpose of this report is to record certain additional findings obtained in the application of the technic previously reported[1] for producing intranuclear inclusion-bodies with substances unassociated with a virus. This procedure consisted essentially of the injection of autoclaved materials subcutaneously into guinea pigs and the subsequent removal of nodules so produced for histological examination. When aluminum hydroxide or alundum was used, intranuclear inclusion-bodies were always found in the mononuclear and giant phagocytic cells of the tissue-reaction about one week after injection and usually as long as the nodules persisted. With ferric hydroxide and carbon they were found but irregularly or in small numbers. Histochemical reactions and morphological characteristics of such inclusions resembled very closely those of viral diseases. However, transmission and other experiments disclosed no virus in association with these inclusions.

Following is a list of additional materials which have been similarly injected subcutaneously into guinea pigs in order to determine whether the resulting tissue-reactions would also contain inclusions: suspensions of normal brain tissue of mice, of guinea pigs, and of rabbits (fresh or autoclaved); normal rabbit tissues (liver, spleen, kidney, testicle); commercial lecithin; alcoholic extract (lipins) of monkey brain; phosphatide of tubercle bacilli;* and 1:2:5:6 dibenzanthracene.† Although the reactions to these substances differed somewhat from one another and from that of aluminum hydroxide, they all contained mononuclear phagocytic cells and in certain instances

[1] Olitsky, P. K., and Harford, C. G., *Am. J. Path.*, 1937, **13**, 729.

* Kindly supplied by Dr. A. L. Joyner.

† Kindly supplied by Dr. A. Claude.

also giant phagocytic cells. Of all the materials, only brain tissue gave reactions containing the characteristic intranuclear inclusions. These bodies were usually few in number and difficult to find, although some sections of the induced nodules contained moderate numbers while in others none were found. Inclusions occurred with rabbit and guinea pig brain, fresh or autoclaved, injected into guinea pigs but not with mouse brain. It was thought that the lipins of cerebral tissue might have caused the inclusions but the results do not show this; the constituent of this material which induces the bodies is still unknown.

It has been shown by Smithburn and Sabin[2] *that the phosphatide of tubercle bacilli, when injected into the connective tissues of guinea* pigs, is ingested by phagocytic cells which can be identified by the supravital staining technic as epithelioid cells. As previously noted,[1] the cells that ingest aluminum hydroxide in the subcutaneous tissues of guinea pigs are also of the epithelioid variety, as determined by the supravital technic. The fact that the epithelioid cells in the phosphatide lesion do not show intranuclear inclusions, while those in the lesion of aluminum hydroxide do show them, indicates that these bodies in the nucleus are not merely characteristic of epithelioid cells in the subcutaneous tissue of the guinea pig but are associated with the presence of certain foreign substances.

That phagocytic cells in sites other than subcutaneous tissue may contain intranuclear inclusions after engulfing aluminum hydroxide is indicated by the following: Aluminum hydroxide has been injected intratesticularly into the peritesticular tissues, and into the pericardium of guinea pigs. Localized nodular masses were formed consisting of mononuclear and multinuclear phagocytic cells actively engulfing the foreign substance. The reactions resembled those in subcutaneous nodules, and the nuclei of the phagocytic cells contained similar inclusions. Although rabbits responded with the same type of tissue-reaction in the testicles as guinea pigs did, inclusions could not be found. Mice receiving aluminum hydroxide subcutaneously showed tissue-reactions similar to those of the guinea pig and the rabbit but in this animal intranuclear inclusion-bodies were seen only occasionally.

[2] Smithburn, K. C., and Sabin, F. R., *J. Exp. Med.*, 1932, **56**, 867.

Dilutions of suspensions of aluminum hydroxide were injected subcutaneously into guinea pigs in order to make a rough determination of how small an amount would produce inclusions. The least amount to give a local reaction with inclusions was that equivalent to 0.01 mg. of Al_2O_3. Alundum is also a form of Al_2O_3 and is commonly used as an abrasive in the preparation of tissues for animal inoculation. In the usual procedure, however, it is completely removed from the tissue-suspensions by centrifugation.

Summary. The following non-virus materials were used in guinea pigs in addition to those already reported: suspensions of normal brain tissue; normal rabbit tissues (liver, spleen, kidney, testicle); commercial lecithin; alcoholic extract (lipins) of monkey brain; phosphatide of tubercle bacilli; and 1:2:5:6 dibenzanthracene. Of these only brain induced characteristic intranuclear inclusions. Aluminum hydroxide injected into other sites than the subcutaneous tissues of guinea pigs produced similar reactions with inclusions. The mouse responded only feebly with inclusions on injection of aluminum hydroxide while the rabbit again failed.[1] The approximate minimal effective dose of Al_2O_3 needed for production of the intranuclear bodies was found to be 0.01 mg. per cc. The various aspects of these findings are discussed.

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PROGRESSION OF DIFFERENT NASALLY INSTILLED VIRUSES ALONG DIFFERENT NERVOUS PATHWAYS IN THE SAME HOST

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In recent years much evidence has been accumulated to indicate that following nasal *instillation* many neurotropic viruses invade the central nervous system (CNS) by the olfactory pathway, and controversy has centered on whether these viruses progress along the perineural spaces or within the processes of the olfactory neurons themselves. Interruption of the olfactory pathway by surgical means has been shown to be capable of preventing poliomyelitis in monkeys induced by nasal instillation of the virus, in spite of the fact that all the other nerves connected with the nasal mucosa remain intact.[1] Similar interruption of the olfactory pathway in rabbits did not, however, prevent the encephalitis which follows the nasal instillation of herpetic virus,[2] and tests for virus during the incubationary period of the disease in normal, unoperated rabbits showed that it was present in the Gasserian ganglia but not in the olfactory bulbs, suggesting that invasion occurred along the 5th nerve.[3] It appeared, therefore, that either conditions were dissimilar in the rabbit and monkey or that different viruses behaved differently. To elucidate this question it was necessary either to vary the virus and keep the host constant or to vary the host and keep the virus constant; it proved easier to do the former.

The viruses of vesicular stomatitis (V.S.), eastern equine encephalomyelitis (E.E.E.), and pseudorabies (the last having been shown to

[1] Schultz, E. W., and Gebhardt, L. P., PROC. SOC. EXP. BIOL. AND MED., 1934, **31**, 728; Brodie, M., and Elvidge, A. R., *Science*, 1934, **79**, 235; Lennette, E. H., and Hudson, N. P., PROC. SOC. EXP. BIOL. AND MED., 1935, **32**, 1444; Howe, H. A., and Ecke, R. S., PROC. SOC. EXP. BIOL. AND MED., 1937, **37**, 125; Gordon, F. B., and Lennette, E. H., *J. Bact.*, 1938, **35**, 43.

[2] Levaditi, C., Hornus, G., and Haber, P., *Ann. Inst. Pasteur*, 1935, **54**, 389.

[3] Levaditi, C., and Haber, P., *Compt. rend. Soc. biol.*, 1935, **119**, 21.

belong to the same "generic" group as herpetic and B virus[4]) were therefore studied in 15-day-old mice (Rockefeller Institute strain). Previous experiments on such mice have shown that following nasal instillation, V.S. virus is found in the olfactory bulbs before it can be detected in the Gasserian ganglia or pons.[5] It is necessary to stress that the nasal instillations were carried out without any mechanical injury to the mucosa, the mice being allowed to aspirate small drops of viral suspension placed on their nostrils. It should also be pointed out that with pseudorabic virus (centrifuged 10% suspension of rabbit-brain in broth) only half the number of mice receiving such *instillations* succumbed (first sign being a persistent and violent scratching of the nose and side of the face), while all mice receiving the same or a smaller dose by subcutaneous, intramuscular, or intraocular *injection* succumbed. Mice of this age were thus found to be much less susceptible to nasal instillation with pseudorabic virus than with E.E.E. or V.S. In the present investigation the pathological method already described in a study on V.S. virus[6] could be used to obtain an indication as to whether the other nervous pathways connected with the nasal mucosa are involved. Partial, serial, transverse sections were taken of the entire skull (without the lower jaw) with all the cranial nerve and autonomic ganglia (except the submaxillary) and superior cervical sympathetic ganglia *in situ*, and also of representative regions of the spinal cord. With the V.S. virus (mice succumbing in 4 to 5 days) and E.E.E. virus (2 to 3 days), the lesions which permitted the mapping of viral progression were those of neuronal necrosis, while with pseudorabic virus (about 54 hours), there was no neuronal necrosis but striking and unmistakable acidophilic, intranuclear inclusions served to indicate the path of the virus.

It is necessary to recall here the chief nervous pathways connected with the nasal mucosa and the situation of the cell-bodies of the neurons of the first and second orders (Fig. 1): (1) the olfactory pathway with the cell-body of the primary neuron in the olfactory mucosa and its *axon* coming into relation with the dendrites of the secondary neuron in the bulbs; (2) the fibers of the 5th nerve whose cell-bodies

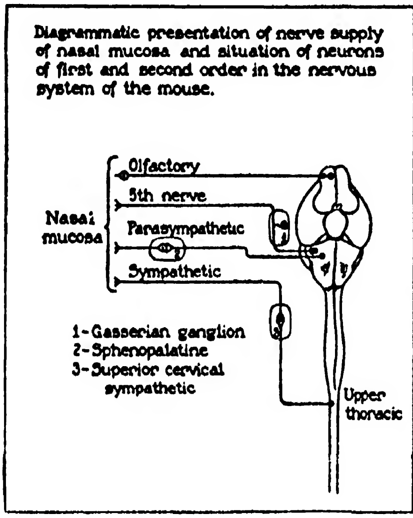
[4] Sabin, A. B., *Brit. J. Exp. Path.*, 1934, **15**, 372.

[5] Sabin, A. B., and Olitsky, P. K., *J. Exp. Med.*, 1937, **66**, 15.

[6] Sabin, A. B., and Olitsky, P. K., *J. Exp. Med.*, 1938, **67**, 201.

in the Gasserian ganglia synapse with the neurons of the sensory nucleus of the trigeminus in the medulla; (3) the sympathetic fibers whose cell-bodies in the superior cervical sympathetic ganglia are in synaptic relation with the neurons in the lateral horn of the lower cervical or upper thoracic region of the spinal cord; and (4) the bulbar autonomic ("parasympathetic") fibers whose cell-bodies in the spheno-palatine ganglia synapse with neurons situated in the region of the 7th nerve nuclei in the medulla.

It has already been reported that following nasal instillation in mice, the viruses of V.S.[6] and E.E.E.[7] invade the CNS along the ol-



Nervous pathways selected by three different viruses in invading the central nervous system after nasal instillation in the mouse.

Nervous pathway	Virus: Vesicular stomatitis	Equine encephalomyelitis	Pseudo-rabies
Olfactory	+	+	−
5th nerve	−	−	+
Sympathetic	−	−	+
Bulbar autonomic (Para-sympathetic)	−	−	+

\+ Lesions present along pathway
− " absent " "

FIG. 1.

factory pathway. In the present study there was no evidence of progression (neither neuronal necrosis nor inclusions) along the 5th nerve, sympathetic, or parasympathetic routes. With pseudorabic virus, however, the reverse was true; specific viral lesions, *i. e.*, characteristic intranuclear inclusions, were abundant in the Gasserian ganglia and sensory nuclei of the 5th nerve, in the sphenopalatine ganglia and region of the 7th nerve nuclei in the medulla, in the superior cervical sympathetic ganglia and the lateral horn of the lower cervical or upper thoracic regions of the spinal cord, but nowhere along the course of the olfactory pathway. In a trial test on an animal

[7] Sabin, A. B., and Olitsky, P. K., *Am. J. Path.*, 1937, **13**, 615 (Abstract).

sacrificed at the onset of nervous signs (which is about 6 hours before death) virus was found (by subinoculation in rabbits) in the Gasserian ganglia and medulla but not in the olfactory bulbs, indicating that in this case the distribution of virus coincided with the observed pathological changes. The following observations support the assumption that the pseudorabic lesions in these structures are the result of the neural progression of virus from the nose and not of any special affinity which virus in the blood might have for them: (a) after injection into the leg muscles there is abundant virus in the blood but no lesions in any of these structures; (b) after nasal instillation of the virus the lesions have been unilateral (in the mice studied thus far), on the same side in all the involved pathways, and none was found in ganglia having no connection with the nasal mucosa. A more detailed description of the localization of lesions in mice after injection of pseudorabic virus by various routes will be given in a future communication, in which it will also be shown that after intraocular (vitreous) injection this virus utilizes pathways (including sympathetic) not employed by the viruses of V.S. and E.E.E. Perhaps the first evidence of the centripetal progression of a virus along the cranial sympathetic system has been obtained in the present study.

It appears clear, therefore, that the facility with which certain neurotropic viruses (*i. e.*, viruses which attack nerve-cells and may or may not be able to attack other types of tissue as well) invade the CNS after nasal instillation is dependent neither upon any supposed connection between the exterior of the nasal mucosa and the subarachnoid space through the olfactory perineural spaces, nor apparently even upon the special position of the cell-bodies of the primary olfactory neurons with their capacity to take up certain instilled substances,[8] but rather upon the special affinity between certain viruses and certain types of cells. In this connection an explanation is required of some recent observations on the fate of a number of viruses instilled into the nose.[8] It was stated that the viruses of St. Louis encephalitis, rabies and louping-ill could not be detected in the "olfactory area" of the brain in less than 24 hours after nasal instillation in mice (the same was found to be true of V.S. virus[5]), while E.E.E. virus

[8] Rake, G., *J. Exp. Med.*, 1937, 65, 303.

was demonstrated within 2 to 5 minutes although not at 6, 8, and 10 minutes or at 6 hours. On the basis of these data, it was assumed that the former 3 or 4 viruses which were called "neurotropic" invaded the nervous system differently from the E.E.E. virus which was termed "pantropic". E.E.E. virus, however, is just as neurotropic as the others and its capacity to multiply in non-nervous tissue elements of mice is shared to a certain extent by the St. Louis encephalitis,[9] louping-ill,[10] and V.S. viruses.[11] It appeared probable that this singular result with the E.E.E. virus may have been due to the fact that a

TABLE I

Does Equine Encephalomyelitic Virus Invade the Brain Within a Few Minutes after Nasal Instillation?

Mice killed at 2 minutes, exsanguinated and nerve tissue removed from cranial cavity within 4 minutes.

Method of removing olfactory bulbs	Dilution of virus and No. of M.C.L.D. instilled	Mouse No.	Test for virus No. of mice injected	No. of mice dead
Special precautions to prevent pulling	1:5	1	2	0
	(200,000,000)	2	2	0
		3	2	0
		4	2	0
Olfactory bulbs pulled	1:100	5	2	1*
	(10,000,000)	6	2	0
	1:1,000	7	2	0
	(1,000,000)	8	2	0

* Virus proved to be cause of death.

given dilution of mouse-brain suspension contains more infective units of this virus than any of the other four; the amount of E.E.E. virus instilled may be estimated at 20 million to 200 million minimal cerebral lethal doses (M.C.L.D.), while the dosage of the other viruses probably ranged between 100,000 and 2 million M.C.L.D. Also it is conceivable that with the larger amount of virus in the nares in the first

[9] Webster, L. T., and Clow, A. D., *J. Exp. Med.*, 1936, **63**, 433.

[10] Fite, G. L., and Webster, L. T., Proc. Soc. Exp. Biol. and Med., 1934, **31**, 695.

[11] Sabin, A. B., and Olitsky, P. K., *J. Exp. Med.*, 1937, **66**, 35.

few minutes (nasally instilled virus has been shown to be rapidly washed away[5,12]) it might be possible to pull up one or more infective units in taking out the olfactory bulbs. An experiment designed to elucidate this question revealed the following: (a) that when the olfactory bulbs were taken out without precautions to avoid pulling, a trace of virus was found in them (within 2 to 4 minutes) when 10 million M.C.L.D. of E.E.E. virus were instilled but not 1 million; (b) that 200 million M.C.L.D. of E.E.E. virus could be instilled without any of it being demonstrable in the olfactory bulbs when they were removed by first being severed from the rest of the brain and then from the olfactory nerves without injury to the cribriform plate and lifted out without any pull on structures in the nasal mucosa (Table I). It would appear, therefore, that there is as yet no evidence that nasally instilled viruses invade the CNS by any direct, open space; the available data seem to point rather to progression along nerve cells and their processes.

Conclusions. Different neurotropic viruses instilled into the nose of the same host (mouse) can select different nervous pathways for invading the central nervous system. The viruses of vesicular stomatitis and equine encephalomyelitis were shown to use the olfactory pathway but not the trigeminus, sympathetic, or parasympathetic pathways, while pseudorabic virus invaded along the latter three routes and not along the olfactory.

[12] Sabin, A. B., and Olitsky, P. K., *J. Exp. Med.*, 1938, 67, 229.

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THE CARCINOGENIC EFFECT OF A PAPILLOMA VIRUS ON THE TARRED SKIN OF RABBITS*

I. DESCRIPTION OF THE PHENOMENON

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PLATES 13 TO 18

(Received for publication, December 2, 1937)

Virus diseases involve reciprocal activities on the part of the infected cells, no matter how soon these die, and their opportunity to influence the situation becomes greater the longer they endure. When they proliferate in series, the resulting growths must be looked upon as the expression of a working partnership with the virus, a state of affairs evident in various tumors of the domestic fowl, and in the infectious papillomas of several mammalian species. Ordinarily these growths, as propagated in the laboratory, are the outcome of infection of the cells of an acutely damaged tissue; and all those caused by the action of any one virus are essentially alike. In the present paper and others to follow it will be shown that tumors of widely differing character will result from the action of a single virus if the cells are appropriately altered before it comes into association with them.

The virus employed for our experiments was that which causes the cutaneous papillomas of western cottontail rabbits (1). On inoculation into domestic rabbits it gives rise to similar growths which often undergo malignant change, carcinomas arising directly from their epithelium (2). This change has never occurred until after several months, in our experience; but various interferences which enhance the cellular proliferation and render it disorderly act to shorten the precancerous period, and they may elicit cancer forthwith when the papilloma has been growing for a long time. Such occurrences have led us to ask whether cancer will develop at once if the virus is brought

* Preliminary note in *Science*, 1936, **83**, 468.

into association with epidermal cells that are already in a pathological state. This is the case, as will be demonstrated.

Rationale

Many agents causing epithelial disturbance and proliferation fail to bring about the tissue alterations preliminary to cancer. For our purpose some substance was needed that would prepare the cells suitably, yet that could be utilized without likelihood that it would itself elicit cancers under the conditions of the experiments. Such a substance was available, namely a tar with which we had done much work, the horizontal retort tar of the Oster-Gasfabrik of Amsterdam.[1] It elicits cancer within a few months in mice (3), and benign "warts" in domestic rabbits (4); yet cancer appears in these latter only after a very long time and then rarely. Our rabbits were tarred on the ears until a few small warts had appeared on some of them, and then were inoculated with virus. Indications had already been obtained of slight differences in the outcome of individual cell-virus associations, as expressed in the growths consequent upon them (5), and hence it seemed important to scatter the virus entities to individual cells altered in varying degree by the tarring. With this in view the virus was injected intravenously. It localized in the tarred skin and here promptly elicited carcinomas as well as a variety of papillomas

Local Effects of the Tarring

The effects of tarring have been often described, yet some account of them as observed in our rabbits is essential. The animals were all agouti (brown-gray) adults weighing about 2500 gm. More than 30 had been utilized previously in an attempt to obtain tar cancers for serological purposes (4), but only one such growth had been got, and this after nearly 2 years, although the tarred skin in most cases underwent the well known changes preliminary to malignancy, numerous "warts" arising, that is to say papillomas and "carcinoids" (6),—growths which look like cancers both in the gross and microscopically, but which remain local, fail to grow on implantation elsewhere in the host (7), and disappear or become mere papillomas if tarring is left off. The experience of others with other tars gives every reason for the supposition that cancer would eventually have arisen in some instances had not nearly all of the animals died of tar intoxication after 6 to 15 months.

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[1] The gift of Dr. Karl Landsteiner.

In the tarred controls of the present work no cancers ever arose. They bring the total number of control animals to more than 90.

The inner surface of the ears was tarred twice a week. In most instances they soon became thickened, warm, and hyperkeratotic, scurfy or macerated; and after 2 to 4 months a few small warts appeared in more than half of the animals. The diffuse histological changes were those often recorded (8), and the growths were the familiar tar papillomas and carcinoids. Not infrequently tumors of the one kind graded into the other.

The later course of events varied widely. The ears of some rabbits remained free from warts, while those of others at the opposite extreme developed numerous growths which, rapidly enlarging, filled the aural shells. Some of the large tumors were predominantly epithelial, and took the form of cauliflowers, cones, or cutaneous horns, but the generality owed their size to proliferation of the connective tissue supporting a thickened, papillomatous epidermis. They often rounded out into tangential spheres, which, as time passed, became pedunculated and underwent retrogression to fibrous tags. Both the papillomas and carcinoids sometimes invaded the connective tissue soon after their appearance, and, extending through lacunae in the cartilaginous plate, formed mounds on the outside of the ear, which occasionally ulcerated; but later they ceased to enlarge and either disappeared or became indolent cones or horns. Often, though growing swiftly at first, they ran a brief course, disappearing despite the continued application of tar. Leroux (9) has well described the retrogression of such tumors.

The rabbits stripped the ears between the paws to remove the tar, and in this way transferred some of it to the outer side, with result that here the skin lost its hair, became hyperkeratotic, and occasionally developed papillomas. Their rarity in this situation during the first 4 months of tarring deserves stress because the introduction of the virus into the blood stream during this period was often followed by the appearance of hosts of papillomas on the outsides of the ears.

In most of the experiments the virus was injected after tarring had been done for 2 to 4 months, and it was kept up for a few later weeks, though this was not essential to the carcinogenesis, as recent findings have shown. When it was finally discontinued the skin of the ears of most of the control rabbits, which had often been macerated, thickened, and furry, rapidly dried down, and a more or less pronounced desquamation took place, revealing at length a smooth, normal-looking surface. Most of the growths dried down too, and some came away; but others, after persisting for a few weeks as mere scabs, began to grow again, becoming horns or dry cauliflowers, or fleshy cones or onion-shaped masses; and sometimes one or more new ones appeared. When they had been large, crowded, and macerating, as rarely happened, they tended to keep on growing, aided by the maceration, and occasionally reached a diameter of several centimeters in the absence of any further tarring. But these large growths were pedunculated, fibrous, and wholly benign.

Very important for the interpretation of the findings after virus injection was the lack of pronounced pigmentation of the tar tumors, and the situation of nearly all on the inside (tarred side) of the ears. In agouti rabbits an occasional small, indolent tar wart is light or medium gray, or very rarely dark, owing to included melanoblasts; but it regularly loses this color in proportion as it proliferates more actively; and vigorously growing tar tumors are never gray but creamy, buff, or pink. Many of the papillomas induced in tarred skin by the virus were by contrast slaty brown, or coal black; and one could be sure that the virus had a hand in any deeply pigmented, yet actively enlarging papilloma which appeared after its injection. This is not to say that virus was absent from such pink, buff, or creamy growths as also appeared, for it elicited many. Furthermore some virus-induced growths that were at first deeply melanotic often became pink later, as happens with such growths on scarified normal skin (2). The appearance during the 3rd to the 5th week after virus injection of numerous papillomas on the outsides of the ears, many of them slaty, was proof positive of the action of the virus.

Our tar was of moderate "carcinogenic" potentialities, to judge from its ability to elicit papillomas. The one cancer it induced, a squamous cell carcinoma with some papillomatous features, appeared after 21 months in an animal tarred for two periods of 5 and 6½ months. When it was killed, after 656 days, a cystic metastasis with the same papillomatous features was present in an auricular gland.

The aim of the tarring after injection of the virus was to prevent reversion of the epidermal cells to the normal state before it had had time to take effect. In skin directly inoculated with a potent virus material 10 days to 3 weeks ordinarily elapses before the first roughening preliminary to papillomatosis can be noted in the gross; but when the individual virus entities are scattered to the tarred epidermis by way of the blood, the growths often appear later and sometimes not until 2 months or more have gone by.

The Virus Materials and Their Effects

Most of the virus materials were generously given us by Dr. Shope, as glycerolated papilloma tissue from cottontails. Attempts to maintain by passage the pathogenicity of active strains of virus from "natural" growths are not very successful, an inoculum of diminished potency being usually obtained from cottontails, while from domestic rabbits the virus is either not recovered at all or in greatly attenuated form. Large amounts of material of high titer were essential to the work, both because of the dilution inevitable to dispersion of the virus on the blood, and because weak strains of it have little carcinogenic capacity, malignancy seldom supervening upon the papillomatosis that they induce (5). The material principally employed consisted of the natural growths from a single cottontail (1211), and it was exceptionally active, as proven by checkerboard titration (4). In the tarred

skin it promptly elicited carcinomas as well as papillomas in a considerable proportion of the injected animals. Another material, nearly as active, was injected in much smaller amount and the virus localizations and cancers were correspondingly fewer. A third material consisted of the pooled papillomas from 7 cottontails experimentally inoculated with a potent virus. It localized in considerable quantity in the ears of the 15 rabbits receiving it, all susceptible and many with tar warts; yet it gave rise only to slowly-growing, ordinary papillomas. This experiment need not be mentioned further.

The Virus Tumors Arising in Scarified Normal Skin

The growths produced by the virus on direct inoculation into the normal skin of agouti rabbits are all papillomas of a single characteristic sort (1,10), some pink but many gray owing to included and stimulated melanoblasts, elements not rendered neoplastic, however. The individual growths take the form of high cones or fleshy onions, more rarely of cutaneous horns or cauliflowers, and their keratin builds high in dry, vertically ribbed or striated layers. In the gross the papillomas resemble some of the tar tumors (11), notably those which continue to enlarge after tarring has been stopped; but as a group they proliferate much more vigorously and are often distinguishable by a pronounced melanosis, a fact already brought out. The cancers arising from them in the ordinary course of events are never pigmented though. They are often multiple, and range in morphology from complicated papillomas of slight aggressive power to the most anaplastic of metastasizing, squamous cell carcinomas.

General Method

In most of the experiments the tar was applied to the central two-thirds of the inner surface of the ears, whence it spread to the edges. Before every third application as much of the old layer was stripped off as possible. A day or two prior to the virus injection all tar was removed, the warts were drawn to size on standard forms, note taken of the general state of the ears, and on the basis of the findings the animals were separated into comparable groups, one serving as control. The charting was frequently repeated later and all significant changes were noted. In most instances tarring was resumed for 14 to 30 days after virus injection, and then the layer was permanently removed and immediate charting done. The controls were similarly treated save that they received no virus and were kept isolated.

The virus suspensions were made by grinding the glycerolated papilloma tissue with sand, suspending in Tyrode (pH 7.0–7.4), centrifuging briefly, and passing the supernatant fluid through a Berkefeld filter. The filtrate was slowly injected into a vein on the outer side of the thigh.

When biopsy specimens were required from the ears, sharp cork-borers were employed and a blow of the mallet.

THE EXPERIMENTS

For the first experiment three groups of tarred rabbits were employed. Into one a virus filtrate was injected, another was set aside for control, while the third received an incubated mixture of virus suspension and heated cancer extract. This was used because Berry has shown that rabbit fibroma virus incubated with heated rabbit myxoma tissue gives rise to disease of the latter type (12). Ours was an attempt, collateral to the main experiment, to convert the papilloma virus into a carcinoma virus. To learn the effect of tarring on ordinary epidermal cells infected with the virus materials, the latter were tattooed into the insides of the ears of several normal rabbits. Tarring was then begun for the first time.

Experiment 1.—The virus-containing fluid was a 4 per cent Tyrode extract of the papillomas from W.R. 1211, which had been passed through one or another of 3 Berkefeld filters, V or N, and been pooled. The cancer extract was made by grinding with sand and Tyrode the tissue of several large, squamous cell carcinomas that had arisen from virus papillomas. The malignant tissue had been frozen and dried some months previously, after separation from all gross remains of the papillomas. A 7 per cent extract of it by dry weight was heated in a water bath at 65°C. for 30 minutes, centrifuged to throw down gross particles, and the murky, supernatant fluid was mixed with an equal portion of virus fluid and incubated at 37°C. for 3 hours prior to injection, while another portion of the virus fluid, mixed with the same amount of Tyrode, was similarly incubated.

Five rabbits were injected intravenously with virus mixed with Tyrode, 4 with the mixture with cancer extract, while 5 more were kept as controls. All had been tarred over the entire inner surface of the ears during periods of from 42 to 89 days, with result in small warts. On the day after the injections, tarring was resumed and repeated twice weekly during the next 25 days.

Two of the rabbits with more numerous and larger warts than any of the others had been placed in the *control group* (Chart 1). The warts of one dwindled during the later tarring but those of the other (rabbit 6) enlarged even after it had been stopped, and new ones appeared. Maceration furthered their course. When this rabbit was killed after 9 months, its ears bore crowded masses of pedunculated, fleshy papillomas, with a few cutaneous horns, all benign. This instance has proved unique in our experience. Little change occurred in the ears of the other controls during the later tarring, and thereafter most of their growths disappeared. In 2 of them a subepidermal mound had formed on the outside of the ear, opposite an active growth on the inside, as if by extension from the latter, but it dwindled and vanished together with this after tarring had been left off.

In a *subsidiary control test* the two virus mixtures were tattooed, with an electric machine, into 2 strips about 5 mm. wide, extending nearly the entire length of the inside of the ears of 5 normal rabbits. The virus with Tyrode was introduced into the left ear, the mixture with cancer extract into the right, and tarring was then begun for the first time and kept up twice weekly until death. Semiconfluent and confluent papillomatosis developed along all the tattooed strips after about 18 days, and the growths enlarged with a rapidity unprecedented in the case of untarred ears (Chart 3). At first predominantly gray, as when no tarring is done, they soon became high, pink, macerating, cauliflower masses. Their foul state led to early death in 3 cases. All of the growths remained ordinary, virus-induced papillomas throughout the 55 to 84 days of tarring, though sections showed their epithelium to have extended down into the profuse reactive tissue, forming cysts, as often happens in the case of vigorous virus papillomas induced in scarified normal skin (2, 5). Elsewhere on the ears the tarring caused the usual hyperplastic thickening, but no warts. In one animal (No. 2), 2 discrete papillomas appeared off the line of tattoo inoculation, but their slaty color marked them as due to the virus.

From Chart 3, of the growths due to *tattoo inoculations* into normal ears which were tarred later, it will be seen that the incubation with cancer extract had greatly cut down the pathogenicity of the virus. It led to no qualitative difference, though, in the growths engendered, all remaining papillomas of the characteristic sort despite great stimulation by the tar.

The course of events in the *animals injected with virus incubated with Tyrode* (Chart 2) differed notably from that in the controls (Chart 1).

During the 3rd week after the injection many new, rapidly enlarging warts appeared, and most of the old ones began to grow at an unprecedented rate (*vide* the records of the 22nd day). In addition, a diffuse change took place in the ears of rabbits 11, 12, and 13. During the 3rd week after the injection they suddenly became much swollen, stiffened, brawny, and hot, and within the next few days papillomatosis appeared over large areas on both their outer and inner surfaces. It took the form of gray and pink, rugose expanses, or of multitudes of minute growths, mostly gray. Some larger, discrete, rapidly growing tumors appeared as well, and many of the preexisting tar warts suddenly began to grow with unprecedented rapidity. The discontinuance of tarring resulted in no slowing of the proliferation, and the ears were soon almost entirely occupied by growths, some of them malignant in behavior. Only the large tumors were charted: there were too many small ones. The time of the first changes after virus injection corresponded roughly with that when papillomas became noticeable in the tattooed

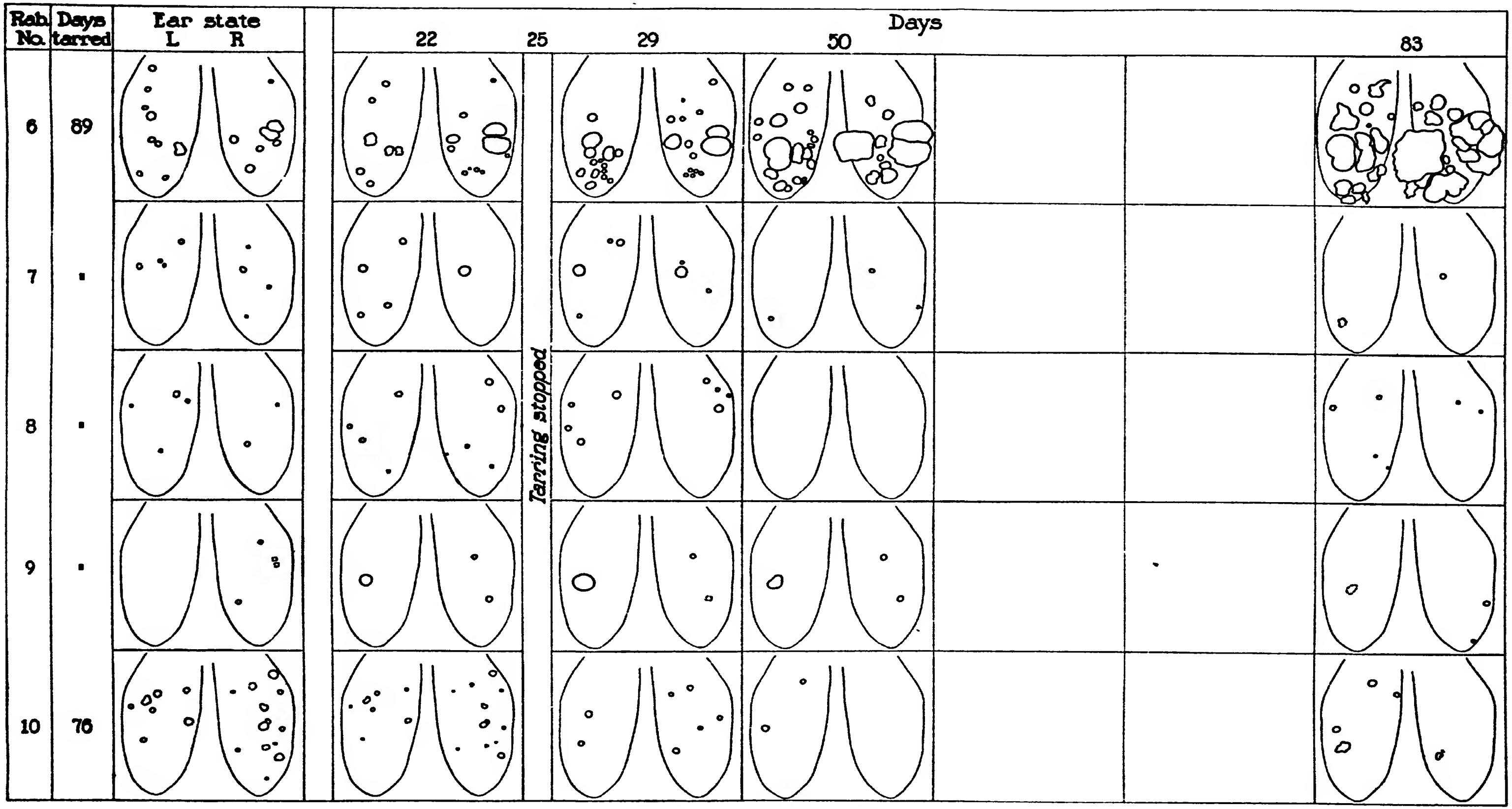

CHART 1. Tarred controls.

CHARTS 1 to 4. Experiment 1. Growths on the inner surface of the ears, before and after virus injection. Minute ones are not charted. Hatching shows areas of confluent proliferation. Along the middle of the ears of rabbits 12 and 13 maceration kept it low, and no charting has been attempted here. Growths of carcinomatous morphology, as determined by biopsy specimens or at autopsy, are shown in solid black from the time when their character was first demonstrated microscopically.

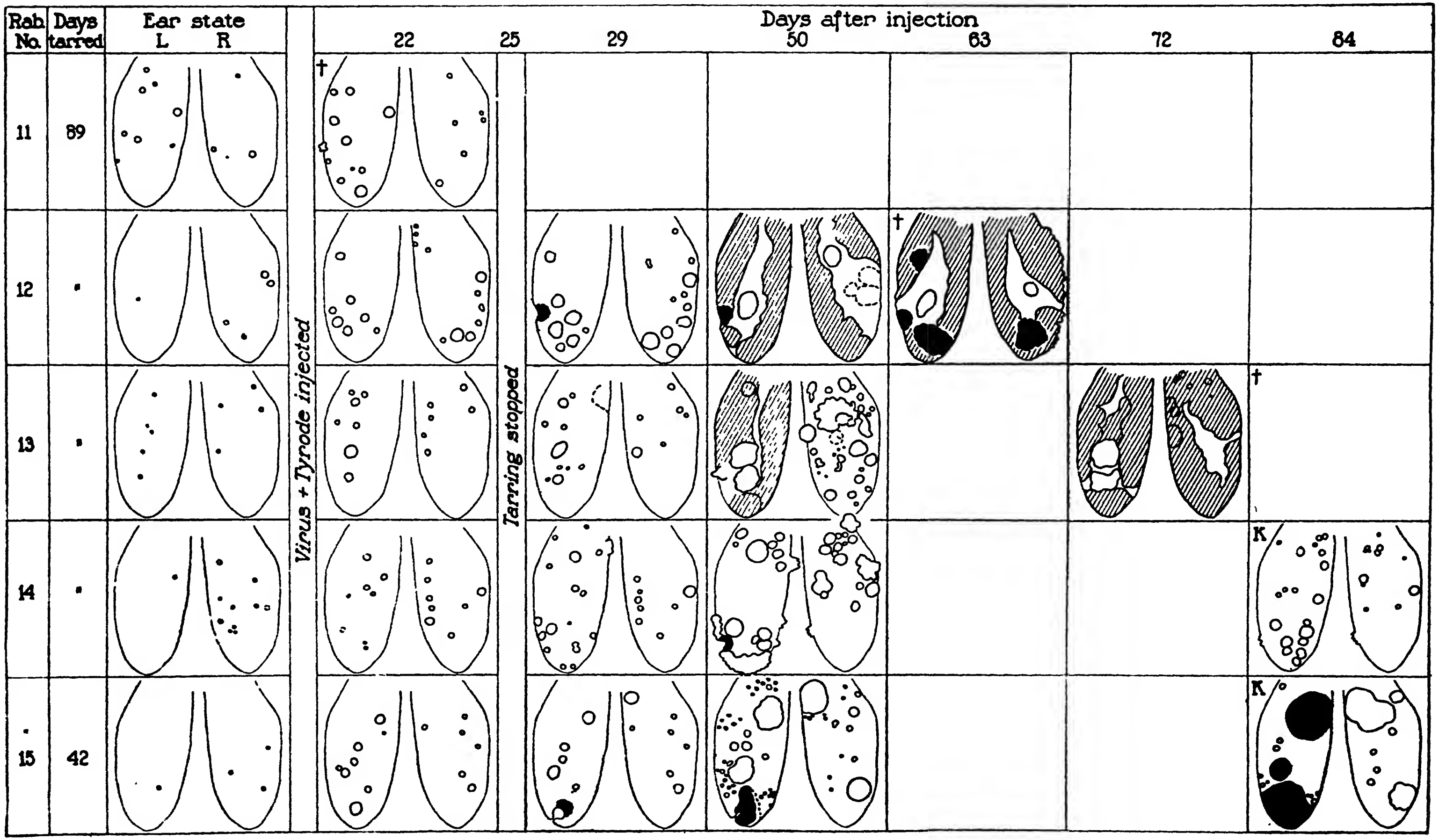

CHART 2. Virus + Tyrode.

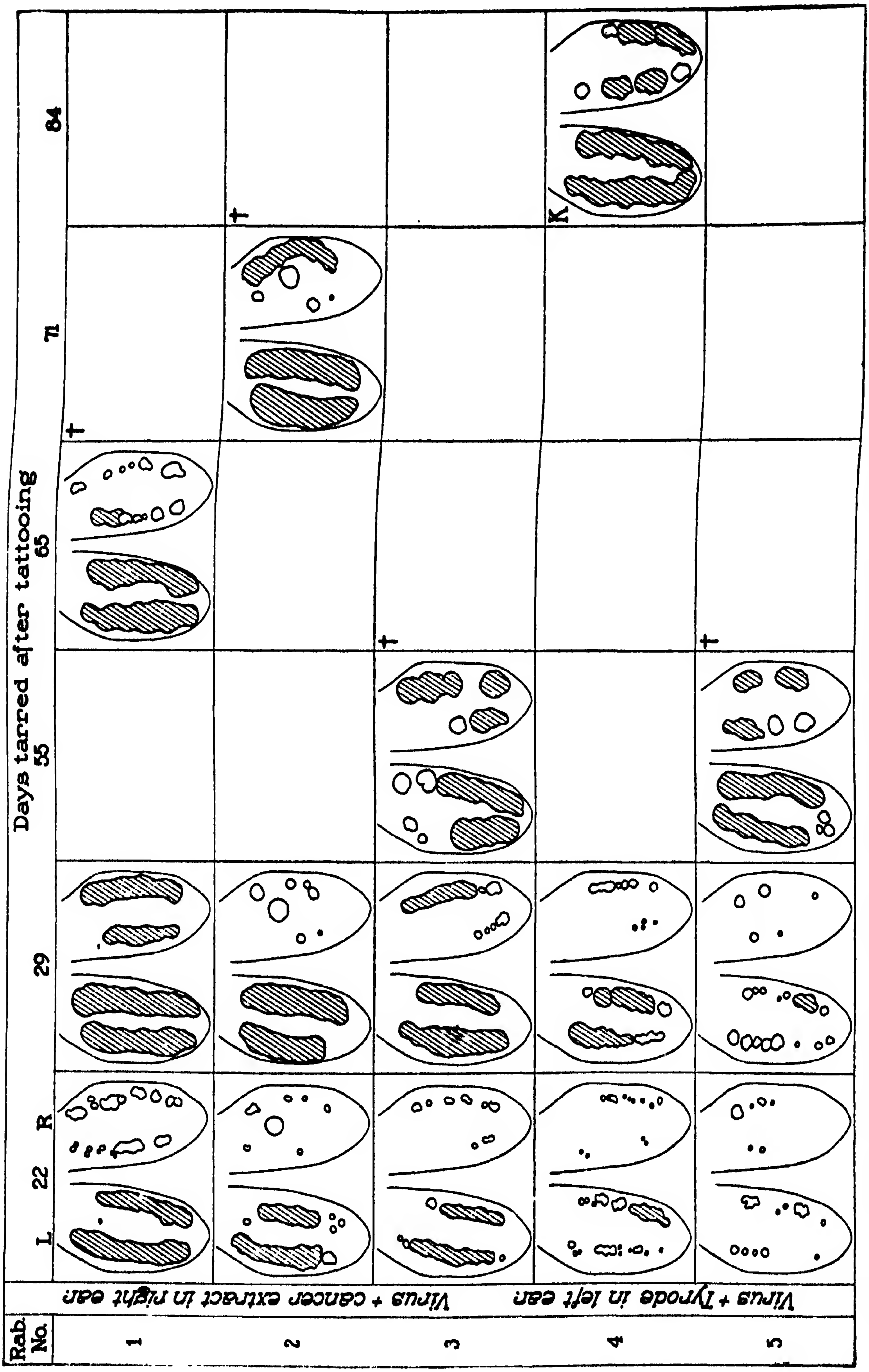

CHART 3. Tattoo inoculation into normal ears that were tarred later. Left ear, virus + Tyrode; right ear, virus + cancer extract.

group. The charts made between the 29th and 50th days have not been reproduced.

Rabbit 11 had thickened, hyperkeratotic ears at time of injection. It died soon after, of intercurrent causes, yet provided much informative material. On the 18th day the ears had suddenly become greatly swollen and brawny; and 2 small, gray, subepidermal mounds had appeared on their outer surface. The swelling increased, more growths appeared, the old grew rapidly (Chart 2), and at death on the 22nd day the ears were from 5 to 10 mm. thick, covered inside with a furry, macerating sheet of fungoid tissue amidst which the old tar warts and some new ones stood forth as slightly higher, discoid growths. Only the latter are indicated in the chart. On section the sheet varied in thickness from 1.5 mm. near the tip of the ear to 7 mm. toward the base, was vertically striated, creamy, streaked and spotted with gray, and consisted microscopically of confluent papillomatous tissue, as if from broadcast, epidermal infection with the virus. The embedded warts, old and new, were also of papillomatous character. Some appeared to be breaking up into squamous cell carcinomas along their base, a frequent finding in ordinary tar warts. Blocks taken at random disclosed occasional localized downgrowths of carcinomatous morphology where no tar warts had existed prior to the injection (Fig. 1).

The skin of the outer side of the ears was irregularly raised, and a cut disclosed numerous separate, discrete, gray or creamy, subepidermal, acorn or onion-shaped growths from 0.5 to 1.5 mm. in diameter. The microscope showed these to be discrete papillomas, deriving from the epithelium of hair follicles and not yet erupted. Some were dark gray.

The early changes in rabbits 12 and 13 were of like sort. The sheets of new tissue which formed on the insides of the ears during the 3rd week after injection soon thickened to 1.0 to 1.5 cm., enveloping the warts previously present or newly appeared, and filling the ears with a high, foul, scabbed mass, save along a central strip where maceration and pressure necrosis kept the tissue low. (Fig. 16 of Experiment 2, illustrates this state of affairs.) Scattered, gray, subepidermal mounds appeared on the outer surface during the 3rd week; and rapidly increasing in size and number these broke through the stretched epidermis, in the case of rabbit 13, and became vigorous, conical or onion-shaped growths with fleshy, bulging, gray bases and dry, sooty, vertically striated peaks,—characteristic virus-induced papillomas in short. Often they coalesced. They were most numerous where the skin was hyperkeratotic and hairless. On the back of the neck, where transferred tar had caused similar, but slighter, skin changes, many discrete or semiconfluent papillomas also arose.

On the 78th day after injection *rabbit 13* died of sepsis. Ruddy fungoid growths up to 2.5 cm. in diameter, some deriving from tar warts, were then present on the inner side of the ears, amidst a thick sheet of foul, vertically striated pink and gray tissue. The microscope disclosed only non-malignant papillomatosis, though the papillomas were of highly various character. Their histology will be

considered in a succeeding paper. Many small abscesses were present in the auricular glands.

The events in *rabbit 12* warrant more detailed description. The ears were slightly thickened and mildly hyperkeratotic at time of injection, and they bore 3 small warts and 2 dubious rugosities. These underwent little change until the 3rd week when some had become raised, granulating discs, while other similar discs had appeared and also ruddy, subepidermal mounds, with scattered gray ones on the outsides. On the 25th day, that of the final tarring, the ears had recently undergone a turgid, hot stiffening. Many new, gray, subepidermal mounds were now present on their outsides, and 4 larger, pink ones had developed there opposite 4 raw, ruddy discs on the inner surface, as if by extension from these. 3 of the inner discs had first become noticeable between the 18th and 22nd days, while the fourth had been present at time of injection, as a small tar wart. Other discrete, ruddy discs or hassocks were also to be noted now on the insides of the ears. On the 29th day the pink, outer mounds had enlarged greatly and 2 were ulcerating. The corresponding discs lay amidst a thick sheet of new tissue covering most of the inner surface of the ear, which sheet is not indicated on the chart. A piece was punched from the disc and mound nearest to the ear margin. It showed what appeared to be an ulcerated, anaplastic, squamous cell carcinoma (Fig. 2), which had extended beneath the adjacent skin, through the ear cartilage, and under a nearby papilloma. During the later weeks, until death on the 63rd day, this growth enlarged but little. The other ruddy, discrete tumors continued to grow however, though obscured by the thick sheet of proliferating tissue covered with heavy, brown scab that rose about them. Under this latter some of them extended widely (Chart 2), while the pink mounds on the outside opposite certain of them became deeply ulcerated. Only the large scabs and the growths discernible through it could be recorded at the late chartings. The numerous, gray, subepidermal mounds on the outside of the ears rapidly enlarged (Figs. 26 and 27) and fused into irregular plateaus covered with breast-shaped, subepidermal protrusions (Fig. 3), each with a nipple-like, dry, black cone at its top. Later sections showed the plateaus to consist of a multitude of keratinizing papillomas of virus type.

On the 50th day a firm, spherical nodule 4 mm. across was felt in a lymph gland at the base of the left ear, the one which carried the biopsied growth. 4 similar growths, with irregular, raised ulcerations opposite them on the outer side, had enlarged progressively; and near the tip of the left ear 2 of these had fused into a thick, fungoid mass.

The animal was sacrificed when moribund, on the 63rd day. The fused growths just mentioned had eaten a foul, transverse furrow, and the ear tip hung limp, attached only by cartilage. The gland nodule had reached 8 mm. in diameter.

Microscopically the 5 aggressive, destructive growths were carcinomas, some anaplastic, others with papillomatous features (Fig. 4). Their extension through lacunae in the cartilaginous plate accounted for the ulcerating mounds on the outer

side of the ears. The numerous other, more or less discrete growths lying amidst the inner masses of fungating, scabbed tissue proved to be papillomas of highly various sorts, none certainly malignant. The greater part of these masses consisted of confluent papillomatosis of ordinary virus type, some of it gray; and the mammillated plateau on the outer side consisted entirely of growths of this kind. Sections from many places were searched for malignant growths that were just arising, but none was found.

The nodule in the basal gland had the morphology of an actively invading squamous cell carcinoma, cystic and with papillomatous features (Figs. 5 and 19). No other secondary growths were found.

The remaining 2 rabbits of the group exemplify the effects of a less abundant localization of the virus in the ears.

Rabbit 15, tarred 42 days when injected, had ears much changed, carrying 3 minute warts. In the 3rd week the growths suddenly increased in number and size. No diffuse thickening or confluent papillomatosis developed. Several of the newly appeared growths were raised, raw discs with depressed centers; and by the 22nd day a pink, subepidermal mound had formed on the outside of the left ear opposite one of them, first noted 4 days previously. On the 29th day, when the mound had ulcerated, a punch biopsy was done, and the growth was found to be a squamous cell carcinoma histologically, which had extended through the cartilage. Later it enlarged rapidly into a broad, fungoid, weeping, lenticular mass on the inner surface of the ear, and a raised ulceration without. So also did 2 similar, discoid growths dating from the 22nd day (Figs. 8, 12). The successive pictures (Figs. 6 to 13) show how quick were the changes. 2 fimbriated, pink growths on the right ear, one of them derived from a preexisting tar wart, also enlarged (Figs. 6, 10). The other, which was new, extended through the cartilage to form a big mound that remained subcutaneous (Fig. 11). In the gross these were mere papillomas. A scattering of discrete, small, sooty or pink growths of the same sort also appeared, notably on the outside of the left ear.

After the 50th day a progressive dwindling took place of many of the smaller growths just mentioned (Chart 2). Few were left by the 84th day, when the rabbit was killed, though the 5 large tumors already described had continued to grow, those of the left ear destructively. The skin between the growths had long since become to all appearance normal. The microscope showed the 3 destructive growths to be squamous cell carcinomas in histology, whereas the 2 on the other ear, though aggressive and somewhat disorderly, were benign papillomas. Many round cells and makrophages were present under and about the small, retrogressing growths, and dark gray spots due to phagocytes crammed with melanin marked where some had disappeared that had been dark gray. There were no metastases.

The ears of *rabbit 14* had been little changed by the tarring. The early events after inoculation were like those in rabbit 15, and soon after tarring was stopped a pink growth appeared which rapidly extended through the ear. Biopsy on the 32nd day showed the growth to be histologically a squamous cell carcinoma. Un-

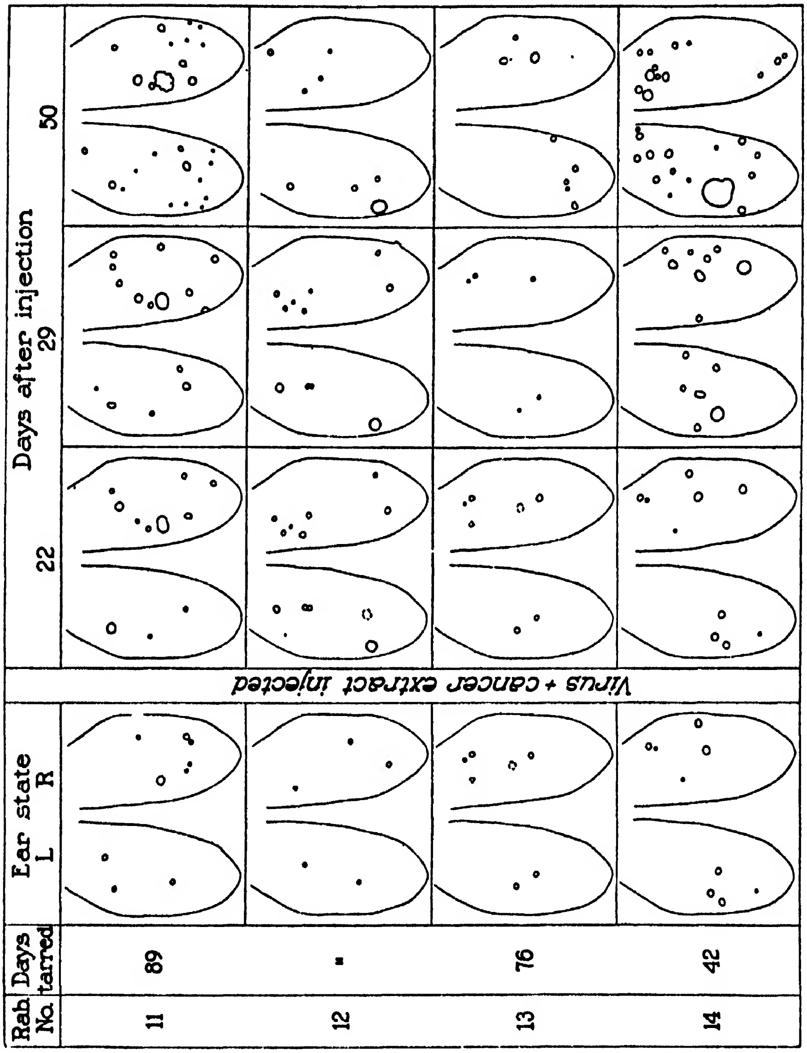

CHART 4. Virus + cancer extract.

fortunately nearly all of it had been taken, and local purulence destroyed the rest. Later some of the fairly numerous growths disappeared, and most of those that persisted to the 84th day, when the animal was killed, were in some degree pedunculated. All were papillomas, and nearly all were dark gray.

In these rabbits, the injection of *virus incubated with Tyrode* was followed by remarkable changes in the ears. After a few weeks, papillomas developed, often in immense number, and in 4 of the 5 animals destructive growths also appeared that behaved like carcinomas and had the morphology of such at early biopsy. They rapidly extended through the cartilage, frequently penetrated into the blood and lymph vessels, and continued to proliferate after tarring had been stopped and even while papillomas on the same ears were retrogressing (rabbit 15). In one animal a secondary growth developed in a lymph node.

The changes were almost negligible by comparison in the animals which received *virus incubated with heated cancer extract.*

In the 3rd week (Chart 4) a few papillomas appeared that were obviously due to the virus, being dark gray, vigorous, rapidly growing, and situated in some instances on the outside of the ears. Others also developed of which there could be no certainty as to cause, since they were creamy or pink. No malignant tumors had arisen by the end of the experiment (84th day), nor did the existing growths alter significantly after the 50th day. Hence the later chartings are omitted.

These findings confirmed the outcome of the tattoo inoculations in showing that the incubation with tumor extract had greatly lessened the pathogenicity of the virus. Indeed the injected animals served as additional controls, testifying to the absence of cancers consequent on the tarring as such.

Sufficient of the virus material of Experiment 1 was available for several more tests. In one the effect was noted of incubation with a cancer extract devoid of inhibitory effect on the virus. The rabbits came from the same batch as those tarred for Experiment 1.

Experiment 2.—A Berkefeld filtrate was made of a 5 per cent extract of material W.R. 1211, and part was mixed with twice its bulk of a heated 8½ per cent extract in Tyrode of a squamous cell carcinoma derived from a virus-induced papilloma. The cancerous tissue had been kept frozen for 16 months. It was ground with sand, extracted with Tyrode, spun to remove particles, and the central portion of the murky, supernatant fluid was taken off through a long needle, and heated at

65° for 30 minutes. The mixture with virus was incubated at 37°C. for 3½ hours and then 13 cc. was injected into a leg vein of rabbits 1-31 and 1-35 N.

In a collateral test of the effect of the cancer extract on the virus filtrate, some of the latter, as such and diluted to 1 per cent, 0.2 per cent, and 0.04 per cent, was mixed with twice its bulk of Tyrode, and with heated and unheated cancer extract respectively, after which it was incubated as above and inoculated into checkerboard squares on the skin of 3 normal rabbits. All of the inocula yielded growths, the heated and unheated cancer extracts having no more effect upon the outcome than the Tyrode. After 9 days papillomas began to appear where the mixtures with 5 per cent virus had been inoculated, and after 16 days and 21 days where 0.2 per cent and 0.04 per cent had been introduced. The number of growths varied directly with the virus dilution.

D.R. 1-31 N, tarred 46 days, had 7 warts, 1 to 6 mm. across when injected with the mixture of virus and cancer extract. Tarring was kept up for 30 days more. During the first 2 weeks after the injection the warts enlarged slowly and no new ones appeared; but within the next 2 weeks a great increase in their size and number occurred (Chart 5). Most of the bigger ones, both old and new, became raised, fungating, ruddy, discoid masses, covered with foul secretion which caused maceration. By the 26th day the largest was 2 cm. across and 5 mm. high. The ears did not dry down after tarring was stopped; the tumors continued to enlarge rapidly, more appearing; and on the 38th day an ulcerated mound was present on the outside of the right ear opposite one of the oldest growths, which had been noted as a slight thickening of the skin at the time of virus injection, and had since become a fungating disc with ill defined borders. Biopsy of it showed a growth with the histology of an anaplastic, squamous cell carcinoma (Fig. 20), which had extended through a lacuna in the cartilaginous plate. It soon caused ulceration on the outer side. Opposite two other, similar discs, first noted on the 24th day after the injection, and now with depressed centers, mounds had appeared, one of which ulcerated in the next few days (Fig. 17). In a region about 2 cm. across, toward the base of the ear, a deep thickening (Y) developed on both sides of the cartilage. A few old growths on the inner surface,—tangential, fleshy spheres or pedunculated cauliflowers, such as tarring frequently elicits,—did not alter noticeably; but elsewhere over this surface great numbers of small, rugose mounds and obvious papillomas arose after the 26th day, and on the outer surface a scattering of subepidermal, breast-shaped mounds, mostly gray, the larger with a central, dark, nipple-like protrusion, consisting of keratinized tissue.

Proliferation continued at a rapid pace, the growths on the inner surface becoming confluent and macerating; and more ulcerating mounds appeared on the outside. Biopsy through one of these latter on the 40th day disclosed another anaplastic, squamous cell growth that had originated on the inner aspect of the ear and extended through the cartilage. The animal, by now very thin, was transfused with 51 cc. of citrated blood on the 38th day. By the 43rd day the shells of the ears were filled with high, fungating masses of growth, blocking the auditory

canals, and covered with thick, dirty brown scab, save along a macerating, longitudinal fold (Fig. 16). The ears were distorted, and nodular on their outer sides

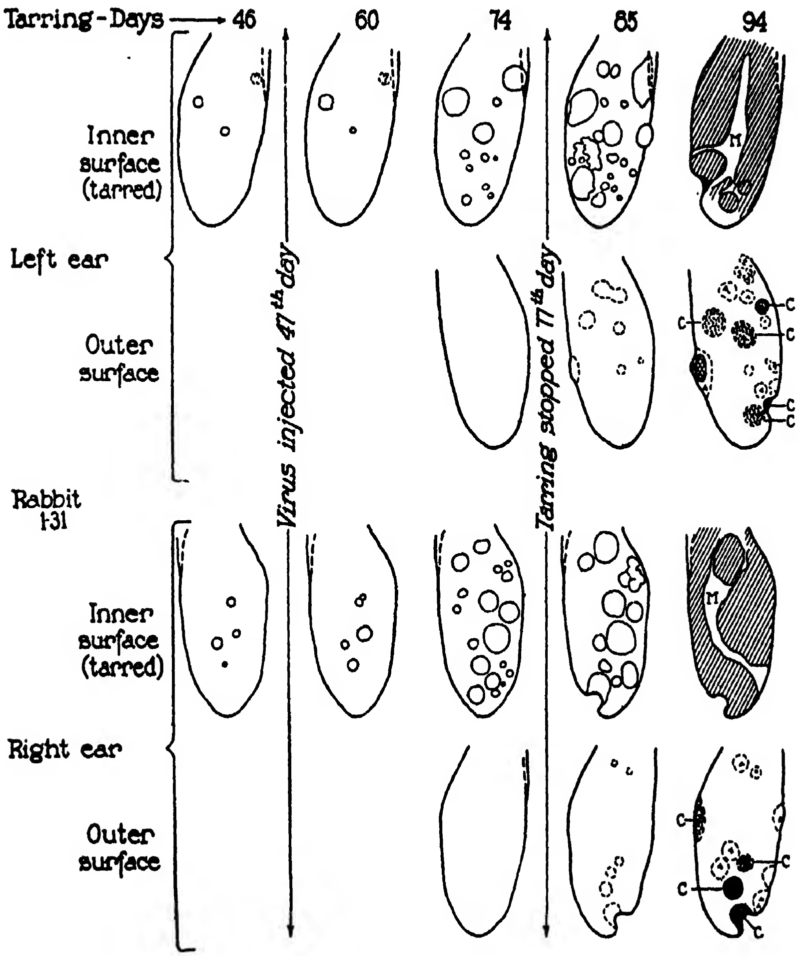

CHART 5. The course of events after virus injection into D.R. 1-31 (Experiment 2). Hatching shows areas of confluent proliferation. The broken lines indicate the ill defined edges of the mounds appearing on the outsides of the ears, cross-hatching there means ulceration, and stippling, subepidermal malignant growths. No attempt has been made to designate the malignant growths on the inner surface, because these were largely hidden in the confluent, fungoid masses. C, C = cancers on the outer surface, as disclosed by sections. M = furrow due to maceration.

(Fig. 17), and here the thickening (Y) was greater, and an ill defined bulge on the outside of the right ear near its base (Cs) first noted on the 28th day, had become prominent, 2½ cm. across, and fluctuating.

The animal was chloroformed on the 48th day, when moribund. On cutting through the ears a thick layer of soft tissue was disclosed, covering the insides, mostly of pinkish cream color but with gray streaks and patches (Fig. 18 *a*). There was some vertical striation but much diversity of texture, owing to imbedded growths, once discrete and still discernible by reason of their irregular markings and serpiginous or dotted necrosis. Many had extended through the cartilaginous plate,—especially near the ear tips where lacunae were frequent,—giving rise to the mounds and ulcers on the outer side. Also present here were scattered, discrete, acorn-shaped growths, creamy or dark gray, with a fine, vertical striation (Fig. 18 *b*),—later stages of the breast-shaped, subepidermal mounds previously noted. They were papillomas such as are caused by the Shope virus, and had originated deep in the skin and dried into blunt, dark cones at their summits. The deep thickening (Y) consisted of papilloma tissue of similar sort that had never erupted.

Blocks were fixed from 20 different situations, mostly where growths had extended through the cartilage. At 12 of them one or more destructive, infiltrative growths were found, squamous cell carcinomas histologically (Figs. 21, 23). All were of considerable size, 1.5 to 4 cm. in diameter, search disclosing none that was just beginning. The bulge on the outside of the left ear (Cs, Figs. 16 and 17) proved to be a lenticular cyst (Fig. 22) full of thin fluid containing necrotic fragments. Its ragged walls were lined with anaplastic tissue of carcinomatous character (Fig. 23), and similar tissue, largely destroyed by purulent infection, was present on the inner surface of the ear opposite it (Fig. 22).

The thick layer covering the inside of the ears consisted for the most part of papillomatous tissue, predominantly of ordinary virus type, though with discrete "papillomas of the second order" (2), cystic papillomas, malignant papillomas, and growths with the morphology of frank carcinomas incorporated in it, as were also a few old, fibrous, more or less pedunculated tar papillomas.

In one of the swollen, auricular lymph glands 2 minute epithelial growths were found, one keratinizing, and of squamous cell type, the other wholly anaplastic. There were many small abscesses in these glands and in the lungs.

D. R. 1-35 N, previously tarred 46 days, had at injection one wart 4 mm. across which did not alter later, though another small growth appeared by the 28th day. Not until nearly 40 days had elapsed did more develop. Then they arose rapidly as numerous, scattered cauliflowers, cones, or onions on the inner surface, and mounds or acorns on the outer side. Nearly all were slaty gray. Tarring was kept up until the 64th day. Under its influence many of the growths became large, and a few on the inner surface discoid and ruddy. On the 81st day the rabbit was killed because moribund from sepsis. The growths had continued to prosper, yet none had extended through the cartilage and only papillomas were found in the numerous blocks taken.

In one of the rabbits of this experiment the injection resulted in a profuse localization of virus in the ears, as attested by the develop-

ment of innumerable papillomas together with many growths that appeared to be cancers and behaved as such during the few weeks before death. Were it not for the results of Experiment 1 and for the findings in the other animal receiving the inoculum, one might be tempted to suppose that the preliminary incubation of the virus with heated cancer extract had resulted in a carcinogenic material of singular potency. In this animal, however, only papillomas occurred, although tarring was kept up for more than 2 months after the virus injection, with the aim of inducing secondary cancerous changes in some of the many, vigorously proliferating growths.

The Changes Caused by the Virus in Skin Long Tarred

It seemed well to extend the observations with the same virus material (W.R. 1211) to rabbits tarred for much longer periods. 2 were available that had been tarred over the entire inner surface of the ears for 40 weeks, and carried in consequence numerous, very large, cauliflower growths and fleshy, rounded tumors with more or less pedunculation, all appearing benign.

Experiment 3.—The rabbits were injected with 15 cc. of 2 per cent filtered virus fluid and two weeks later tarring was stopped. *D.R. 6-46* had at injection 15 warts up to 3 cm. across and more than 2 cm. high. None was gray. 17 days later a few gray, subepidermal mounds had appeared on the outer sides of the ears, and the biggest growth on the inside, previously a smooth, ruddy, almost tangential sphere, showed several protruding bosses on its surface, which in another 3 days had become large, rugose, and patched with gray (Fig. 30). Many small, gray papillomas had appeared elsewhere on the inner side of the ears, and on the outer side at one edge a scabbed thickening had developed, more than 1 cm. away from any other lesion. After another 4 days this had become a raised, ulcerated disc, 1.3 cm. across, with a smaller, slightly raised scab opposite it on the inside. It looked cancerous.

During the next 7 weeks the new growths on the insides of the ears enlarged rapidly, became crowded and in some regions confluent. Where discrete they were cone- or onion-shaped, gray or infrequently pink. They encroached upon and obscured all of the tar warts except the large pedunculated sphere studded with bosses. This was snipped off on the 36th day, and was found to consist mainly of fibrous tissue, covered almost everywhere with thick, papillomatous epithelium, some of it melanotic. There were some gray or creamy "acorns" embedded in the growth, and in a few places its covering was mere hyperplastic

epidermis. Many gray papillomas had appeared in the hyperkeratotic skin on the back of the neck.

The rabbit was killed on the 73rd day after injection, while still in excellent condition. The growth that looked cancerous had ulcerated further, causing necrosis of the cartilage and a deep nick in the ear. It had extended to a distance of about 1.3 cm. from this nick on either side of the cartilage, and was erosive, not fleshy. Microscopically, it was a squamous cell carcinoma, anaplastic in some regions. No malignant changes were evident elsewhere. Both ears were almost completely covered on the inner surface with pink or gray growths, and many of the latter were present on the outer surface as well. The regional lymph glands were not examined.

D.R. 6-51 had six large, pink, tar tumors at time of injection, several of them 3 to 4 cm. in diameter, fleshy and pedunculated. When tarring was stopped 14 days later, some scattered, small, gray mounds had just appeared on the outer surface of the ears; and in another 3 days these organs were noted to have undergone the brawny, hot thickening and stiffening already described. A raised, scabbed disc had formed in the hyperkeratotic skin of the outside of one ear, more than 2 cm. away from the nearest growth, and a mound had appeared opposite a tar tumor on the inside. The surface of several of the larger tar tumors had become nodular, and elsewhere on the inner surface many new, pink, discoid tumors had arisen. All enlarged rapidly. By the 21st day the ears were greatly thickened, distorted, hot, heavy, and pendulous. The changes continued; the ears became several centimeters thick; nodular bulgings appeared on their outer surface, subepidermal growths along their edges (Fig. 25); their orifices became choked with pultaceous matter, and their concavities full of confluent growth, partly obscuring the tar tumors (Fig. 24). On the hyperkeratotic back of the neck numerous gray papillomas appeared.

The animal died of seropurulent pleural effusions on the 32nd day. The ears weighed 220 gm. (Fig. 25). On the inner side of the cartilage was an irregular sheet of vertically striated tissue 8 to 12 mm. thick; and a similar sheet, mostly subepidermal, existed outside. Most of the old tar tumors had been destroyed by maceration. The scabbed disc had grown smaller: it proved due to a papilloma of virus type. No malignant growth was found elsewhere on extensive sectioning.

The virus localized abundantly in the ears of these rabbits. In view of the marked general changes, the numerous, large, tar warts, and the outcome of the previous experiments, it had seemed reasonable to suppose that many malignant growths would be elicited. On the contrary, only one appeared after the injection. Yet the conditions were so favorable to the virus, that the ears of one animal reached enormous size within a few weeks, owing to confluent papillomatous proliferation.

Later Course of the Induced Malignant Tumors

Most of the rabbits in which the virus caused confluent or coalescing growths died early from sepsis incidental to their presence. Metastases had already developed in the lymph nodes of 2, but they might have arisen from cells thrust into the lymphatics when the ears were biopsied. In the next experiment the inoculum was reduced to elicit fewer growths; and these were allowed to run their course without interference. The virus material W.R. 1211 was by now exhausted, and recourse was had to that from W.R. 1183.

Experiment 4.—The 42 rabbits had been tarred for 89 days over about half of the inner surface of the ears. At injection they were separated into four comparable groups. The ears of some were but little changed, and carried no warts or only one or two; but those of more than half of each group were thickened, hyperkeratotic and hot, and bore several warts.

Eight rabbits were injected intravenously with 15 cc. of 0.5 per cent filtered virus fluid, and some of it was tattooed into an area 2 to 3 cm. across on the shaved, left side of the body. Tarring was kept up for 25 days more. All of the tattoo inoculations yielded papillomas, and in 5 of the 8 animals virus localized in the tarred skin as proven by numerous sooty papillomas on both aspects of the ears. In 3 of the 5 many rapidly enlarging, ruddy tumors also appeared on the inner surface, and in 2 of the 3 some of the growths here were malignant. A second group of 9 rabbits were similarly injected but tarred no longer. Though they were susceptible, as shown by growths at the tattoo sites, relatively few sooty papillomas developed on their ears, only occasional ruddy growths, and no malignant tumors. A third group, of 11 animals, received 0.2 per cent virus and were tarred later, like the first lot. Though they all developed tattoo growths very little virus came out into the ears, few gray papillomas arising, only an occasional ruddy growth, and none that was malignant. This group can be thought of as furnishing accessory controls. The 10 controls proper were tarred like the first and third lot and most of them were kept for months after the termination of the experiment. Their warts remained small and either disappeared later on, persisted as such, or very slowly enlarged.

In a succeeding paper, the findings will be scrutinized in detail. Here only those animals of the first group that developed malignant growths need be considered. A staphylococcus meningitis caused death of one (No. 28) on the 71st day. It had no warts when injected, nor any 2 weeks later, but during the 3rd week, when virus papillomas were appearing on the side, many discrete growths arose on the ears, and after another 2 weeks more than 50 were present, mostly sooty and still small. At the charting of the 36th day a new, pink, subepidermal mound attracted attention. It was 6 mm. across, had encroached upon a neighboring, sooty papilloma, appeared to be infiltrating laterally, had a scabbed top,

and was recorded as probably malignant. A week later a firm mound had developed opposite it on the outside of the ear. It grew steadily, infiltrating and ulcerating on both aspects of the organ, and when the animal died was 2 cm. in diameter (Fig. 28). On the outer side several fleshy, subepidermal prongs, not visible in the photograph, extended from it toward the base of the ear. Such prong-like extensions have never been found extending from the tar carcinoids of our many control rabbits, nor have these grown after tarring was stopped. The microscope showed a growth with the histology of a squamous cell carcinoma with elongated, cystic extensions such as are encountered in many cancers derived from ordinary, virus-induced papillomas (5). It will be pictured in a later paper.

The other rabbit developing malignant tumors (No. 27), had 3 small warts at injection. During the 3rd week thereafter growths suddenly appeared in considerable number on the ears, nearly all on the left, some pink, the majority gray; and papillomas were now visible at the tattoo site. During the next 2 weeks many growths developed on the left ear and a few on the right. On the 36th day a firm thickening could be felt on the outer side of the right ear opposite a fleshy disc with ill defined margins that dated from the 3rd week after injection. The disc soon became a broad, weeping mound which encroached upon and undermined the nearest growth, a sooty papilloma originally more than 1 cm. away (Fig. 31); and the extension to the outer side of the ear, elsewhere wholly devoid of tumors, underwent ulceration. The ulcer had raised, infiltrating edges (Fig. 32). At the 123rd day the malignant growth had long since destroyed the neighboring, sooty papilloma on the inside, as well as others further off and a considerable part of the ear itself (Fig. 33). The foul, granulating expanse was stippled with yellow dots, suggestive of keratinization. The ear had reverted to the normal in the region not occupied by growths.

The enlarging tumors on the left ear soon became crowded, and maceration took place along its middle. Here, where the tar had been directly applied, no gray growths arose but instead several weeping, pink discs that rapidly grew large and fleshy. Owing to later distortion these came to occupy the bottom of a deep, longitudinal fold, with many high, crowded, gray papillomas fencing them from close inspection. On the 70th day a mound had appeared outside the cartilage, opposite one of them, and a week later 2 more mounds opposite others. By now many large, conical or jagged papillomas, more or less confluent and mostly sooty, were present on both sides of the ear.

On the 93rd day the lymph glands at the base of both ears were enlarged and firm, and 2 weeks later a nodule nearly 1 cm. across was palpable in a gland on the left, now 3 cm. long, and another on the right, 0.5 cm. in diameter. The nodules enlarged rapidly and others appeared in neighboring glands. Figs. 14, 33, and 34 tell the state of affairs on the 123rd day. The ears had undergone extensive destruction. By the 156th day about half of the right one was gone (Figs. 35, 36), and only the stump of the left remained, thickened, brawny, and edematous. Enormous metastatic masses had replaced the auricular nodes, and others

existed lower down in the neck. A thick cord could be felt connecting the growth on the right ear with the nearest glandular mass. The skin over the most prominent of those on the left side was attached, and fluctuation could be felt immediately beneath it.

The thin, weak animal was killed on the 159th day. Microscopically the destructive growths on the ears had the morphology of more or less anaplastic, squamous cell carcinomas (Fig. 37), and the masses in the neck consisted of similar tissue, with remnants of the lymph nodes and of the adjacent salivary glands (Figs. 15, 29), amidst abundant, reactive connective tissue. The fluctuation was due to an abscess amidst the neoplastic tissue. The cord connecting with the growth on the right ear was carcinomatous. No visceral metastases were found, but much amyloid change in liver and spleen.

In this experiment reducing the amount of virus injected had one of the desired results, premature death from sepsis being much less frequent; but the reduction was carried too far in one group of animals, with the result that very few virus localizations occurred in their ears. When many took place the papillomas still appeared relatively late and grew rather slowly as compared with those of the previous experiments. 5 of the 8 rabbits receiving the largest inoculum and tarred for 25 later days developed sooty growths on the ears in moderate number, with some pink ones; and concurrently malignant tumors appeared in 2 of the 5 individuals. One died early of intercurrent causes. A notably invasive growth arose on the right ear of the other, as also a few sooty papillomas, while on the left ear several malignant growths of the same sort appeared, together with numerous papillomas. Both ears underwent progressive destruction and immense metastatic masses formed in the regional glands. The associated papillomas remained merely such while this was happening.

The Effect on Tar Warts of Virus-Induced Fibromatosis

Castiglioni (13) has reported that rabbits rendered syphilitic by the intravenous route are notably responsive to tarring, although the ears show no signs of syphilitic infection. He describes both papillomas and carcinoids as appearing early, but no cancers arose. It has seemed possible that some of the growths elicited in the present work might be tar warts stimulated to factitious malignancy (14) by connective tissue disturbances referable to the virus, although no such disturbances have been found, and the hypothesis will not explain the

metastasizing tumors or those which continued malignant while round about them the ears were reverting to normal. Nevertheless tests were undertaken with the virus causing rabbit "fibromas" (15) to find what effect a vigorous connective tissue proliferation would have upon tar warts.

A 5 per cent extract of glycerolated "fibroma" tissue[2] in Tyrode was cleared with the centrifuge, and 0.1 to 0.3 cc. was injected at each of 4 to 16 situations in the skin of the inner surface of the ears of 4 rabbits. The ears of 3 had been tarred twice a week for 165 days, and carried 4 to 7 warts from 3 to 25 mm. in diameter, while those of the fourth had been tarred thrice weekly for 5 months, and, after 6 months' intermission, for 5 months more twice weekly, with result in 29 warts 3 to 10 mm. across. The virus fluid was injected directly under or next to several warts of each animal, and the tarring was kept up afterwards for 4 weeks. Early in the 3rd week "fibromas" developed as ruddy mounds or discs, but no new warts were evoked. Repeated punch biopsies were made. Often the fibromatous proliferation took place immediately under the epithelium of the tar tumors, yet the latter did not extend downwards, the only effect upon them being pressure distortion or occasional local necrosis. Frequently the "fibromas" extended through the cartilaginous plate, with result in low mounds beneath the outer skin. Here too no epithelial downgrowth was induced.

The Findings as a Whole

The present paper is mainly concerned with the gross changes taking place in the tarred skin after the virus lodged in it, and with those tumors which appeared to be malignant. A variety of other growths were also elicited in addition to the papillomas characteristic of the virus, namely papillomas of complicated pattern and problematic malignancy, cystic papillomas, and frankly malignant papillomas. They will be considered in a later paper. The unavoidable employment of several virus materials added a complicating factor to the many implicit in the experiments; yet the results are consistent.

In the individuals most susceptible to the virus, its localization at numerous situations in the tarred ears was signalized by a sudden, brawny, warm thickening which occurred in the 3rd week after the injection, an incubation period roughly corresponding with that when a virus fluid of moderate pathogenicity is rubbed into scarified normal skin. During the next few days a more or less confluent

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[2] Strain D, D.R. 1514, kindly provided by Dr. Shope.

sheet of papilloma tissue formed on the inside of the ears, and low, scattered, subepidermal mounds, mostly gray but occasionally pink or creamy, appeared in the hyperkeratotic skin of the outer side. At the same time some of the preexisting tar warts, previously indolent, began to enlarge with great rapidity, and fleshy, pink or cream-colored growths also arose where no warts had been present. That these were nearly all referable to the action of the virus was plain from the findings in the controls; yet all resembled in their gross appearance one or another of the various tumors that follow merely upon tarring, though they proliferated much more vigorously. Some were mounds or discs, fleshy cones or dry, cutaneous horns; others were of cauliflower, onion, or hassock shape; while yet others became fleshy, fibrous spheres that underwent pedunculation secondarily.

In many cases the mounds forming on the outside of the ear rapidly became breast-shaped, with nipple-like, dark, keratinized protrusions which later heightened into vertically striated cones, the growths then taking on the aspect of ordinary, virus-induced papillomas. In some animals, though, they remained predominantly subepidermal, and, coalescing, formed plateaus covered with rounded protrusions (Fig. 3). Many growths on the inside of the ears were lost to view in the sheet of papillomatous tissue that rose about them, but others maintained their identity until the aural shell filled up with foul, macerating tissue and its whole interior became a fleshy, scabbed mass (Figs. 16, 17). Then, on cutting through the ear, the sheet of new tissue showed some vertical striation, perhaps streaked or mottled in gray, the pink growths amidst it presenting a diversity of patterns (Fig. 18 *a*).

Special significance attaches to these rapidly enlarging tumors which took the form of low mounds or raw, beefy discs dotted with yellow necroses; for many of these proved malignant. They had ill defined margins, or a rim of raised and infiltrated skin, and sometimes a depressed, crateriform center. Some were derived from tar warts, but others arose where none had been visible. As a rule they enlarged progressively, even though tarring was stopped; invaded and replaced the tissue about them; and soon extended through the cartilage, causing ulceration on the outer side.

Death occurred early, owing to the septic state of the ears, which were full of foul, fungating growth, yet metastasis had already occurred in some cases. In one of these the secondary nodule was recognized only 32 days after the parent growth on the ear had first declared itself, 18 days after virus injection. Both grew rapidly.

When less virus localized in the ears, as attested by a smaller number of sooty papillomas, the growths of other sorts were less numerous. They arose concurrently with the crop of papillomas, and at about the same time some of the tar warts started to grow at an unprecedented rate. The appearance of one or more gray, subepidermal mounds on the outer surface of the ears often served as a tell-tale to the presence of the virus when it was proving effective at but few situations. In such instances some gray or almost black growths usually arose on the inner

surface as well, but here the melanoblasts responsible for their hue (16) were less frequent and the epidermal alterations were greater. The more pronounced these alterations the more frequent in general were non-pigmented growths.

Generally speaking, malignant growths were few as compared with ordinary virus-induced papillomas; and in proportion as the amount of virus acting upon the ears was diminished, they became rare. One noteworthy exception to the rule was met, however, several cancers arising in rabbit 15 of Experiment 1 at the same time as did thinly scattered papillomas (Figs. 6 to 13). Only animals favorable to the virus, as evidenced by the induced papillomatosis, developed malignant tumors also; yet they failed to occur in some individuals so favorable that their ears were largely converted into papillomatous masses (Figs. 24 and 25). The malignancy was often multiple, and involved both ears.

The stimulating effect of tar upon the growths that it elicits is one of the truisms of cancer research. Virus-induced papillomas are also very responsive to its influence, often burrowing, extending through the cartilaginous plate, and ulcerating, though remaining essentially benign, as shown by the fact that they build up later into discrete conical or onion-shaped growths of the characteristic sort. Needless to say the tarring after virus injection must have elicited some tumors referable merely to it, though few such developed in the controls. The contrast afforded by these latter animals was remarkable.

A secondary resistance to the sooty papillomas occasionally developed, as evidenced by the retrogression of most of them; and under its influence some of the pink growths that had been growing rapidly also disappeared, or reverted to their previous indolent state. This happened in rabbit 15 of Experiment 1. Yet while most of the papillomatous growths elicited by the virus in this animal were disappearing the malignant tumors continued to proliferate and invade, and a few papillomatous growths also went on enlarging rapidly (Chart 2).

Some tar usually reached the base of the neck where the ears rested, producing alopecia and hyperkeratosis; and many growths sometimes

appeared there after the virus injection. They were always gray or pink papillomas of the characteristic sort.

DISCUSSION

Were the malignant growths which arose in the tarred skin referable to the action of the virus? In only one of more than 90 tarred but uninoculated rabbits, of the breed experimented upon, many of them tarred for long periods, has any such growth arisen. It was a metastasizing squamous cell cancer consequent upon tarring for two periods of 6 months and 5 months respectively, and it appeared after 21 months in all. When the virus failed to localize in the ears of the injected rabbits no malignant growths developed; and the more abundant its localization as evidenced by gray papillomas engendered, the more often, generally speaking, did they occur. They appeared at the same time as these papillomas, that is to say a few weeks after injection of the virus; and they arose only when conditions were favorable to the latter, as demonstrated by the behavior of the gray growths. They occurred with a frequency unparalleled in the recorded experience with tarred rabbits, and were often multiple, and frequently numerous, facts which will find further exemplification in a succeeding paper. In sum, the facts leave no doubt that the virus called them forth.

Were the malignant growths the expression of an unique disease, simulating cancer but to be discriminated from it? A categorical answer can be given to this question, owing to the fact that tumors as a class inscribe themselves upon the organism with a minute elaboration. The malignant growths of the present work exhibited all those histological features which typify carcinomas of squamous cell origin; yet such features do not suffice in themselves for a diagnosis of cancer in the case of growths arising in tarred skin. These may be highly anaplastic and invade rapidly, penetrate through lacunae in the cartilage and cause ulceration, yet disappear after a time or undergo a transformation into indolent papillomas, even when tarring is kept up (9). Some of the tar tumors of our control animals were of this sort, incapable of independent malignancy, as shown by their disappearance or reversion to the benign state when tarring was left

off. Final proof that the malignant growths evoked by the virus were true carcinomas rests upon those cases in which the growths not only had the morphology but manifested the independent activity of such tumors. This happened in every instance save one, when the animal lived long enough, and in this one the growth was destroyed by purulence after biopsy (Experiment 3).

Since the publication of a preliminary report of the work here set forth, Lacassagne and Nyka (17), using benzpyrene instead of tar, have confirmed its findings, and Andrewes, Ahlström, Foulds, and Gye (18) have studied the alterative effects of tar upon the outcome of infection with the virus which gives rise to rabbit "fibromas." Ordinarily this virus causes connective tissue growths which are restricted to the immediate site of inoculation and regularly retrogress after a few weeks of active proliferation. This was the case with the strain employed by the authors mentioned. But when the animal had received an intramuscular injection of tar, and the virus was thrown into the blood stream, not only did it elicit in some cases widely distributed growths with the character of "fibromas," which enlarged progressively and caused death, but in certain instances growths arose which the authors describe as of neoplastic character. They appeared at the site of the tar injection, that is to say, where the connective tissue had undergone most change. In this general relation it is important to recall the demonstration of Teague and Goodpasture (19) that the virus of herpes simplex, when acting upon tarred skin, induces lesions resembling those of herpes zoster.

SUMMARY

The Shope papilloma virus elicits carcinomas forthwith, as well as papillomas in great variety, when it is distributed by way of the blood stream to the tarred epidermis of domestic rabbits.

The phenomenon will be analyzed in succeeding papers with the aid of additional instances.

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EXPLANATION OF PLATES

All of the sections were stained with methylene blue and eosin.

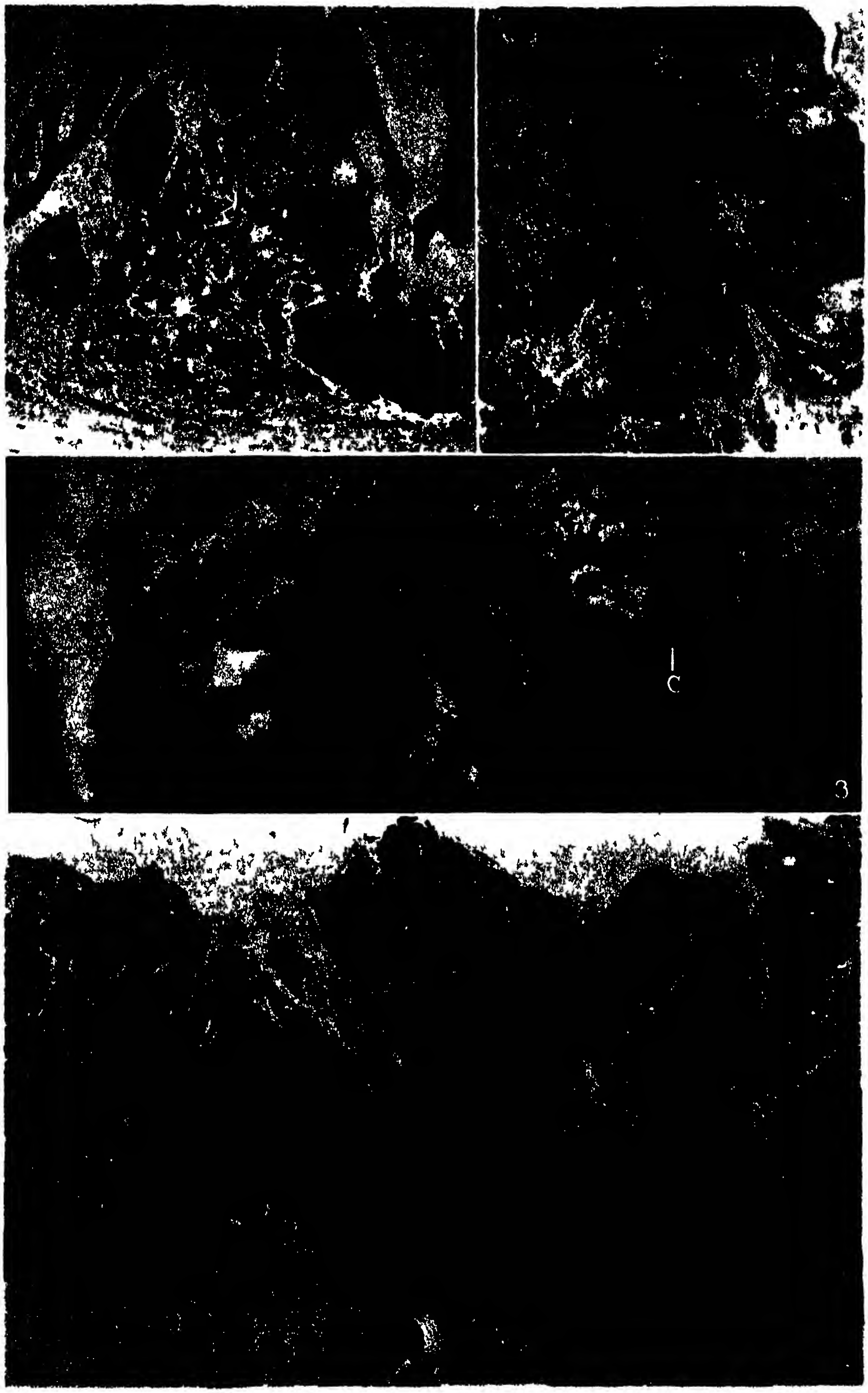
Plate 13

Fig. 1. Early malignant downgrowth in rabbit 11 of Experiment 1: 22nd day after inoculation. The surface epithelium from which the anaplastic proliferation derives, though still intact, stains much lighter than that adjoining it. ×44.

Fig. 2. Ulcerating, destructive growth at the ear margin of rabbit 12: biopsy specimen of the 29th day. The anaplastic proliferation has extended beneath the skin, past the cartilage, and under an adjacent, newly appeared papilloma at the ear edge. ×12½.

Fig. 3. Ears of rabbit 12 on the 43rd day after virus inoculation: to show some of the malignant growths (C, C), and the mamelonated plateaus of papillomatous growth, largely subepidermal. The malignant ulceration has eaten deep into the tip of the left ear. (The aural shells were full of coalesced, fungating tissue,—*vide* Fig. 16 of another animal.) ×2/5.

Fig. 4. Autopsy specimen from rabbit 12: Part of a large, ulcerated, malignant growth with papillomatous features. Extension has taken place through many lacunae in the cartilage. At the right there is heavily pigmented, benign papillomatosis. The keratin overlying it has been cut away. ×11.



Photographed by Joseph B. Haulenbeek and Louis Schmidt

PLATE 14

FIG. 5. Edge of a cystic, metastatic growth in an auricular node of rabbit 12. (See also Fig. 19.) $\times$ 78.

FIGS. 6 to 13. The ears of rabbit 15. Figs. 6 and 7, 8 and 9 show the inside and outside of the right and left ears respectively, on the 41st day. Figs. 10 and 11, 12 and 13 show the state of affairs 16 days later. The hole in the left ear (Fig. 8) was due to a biopsy on the 29th day. It later filled with growth (Fig. 12). C, C = carcinomas, as determined microscopically. P, P = the aggressive papillomas described in the text. The scattered small growths were all ordinary papillomas, mostly dark gray.

FIG. 14. Ears of rabbit 28 (Experiment 4) on the 123rd day (see also Figs. 33 and 34). The ulcerated, coalesced, malignant growths on the left ear, which have extended through and destroyed its central portion, are surrounded by numerous, discrete, sooty papillomas. A similar malignant tumor has perforated the right ear and extended to the outer side which is devoid of growths elsewhere. There is metastatic enlargement of the auricular lymph nodes (arrows). $\times$ 1/3.

FIG. 15. Metastasis in an auricular lymph node of the same rabbit. $\times$ 41.

Photographed by Joseph B. Haulenbeck and Louis Schmidt

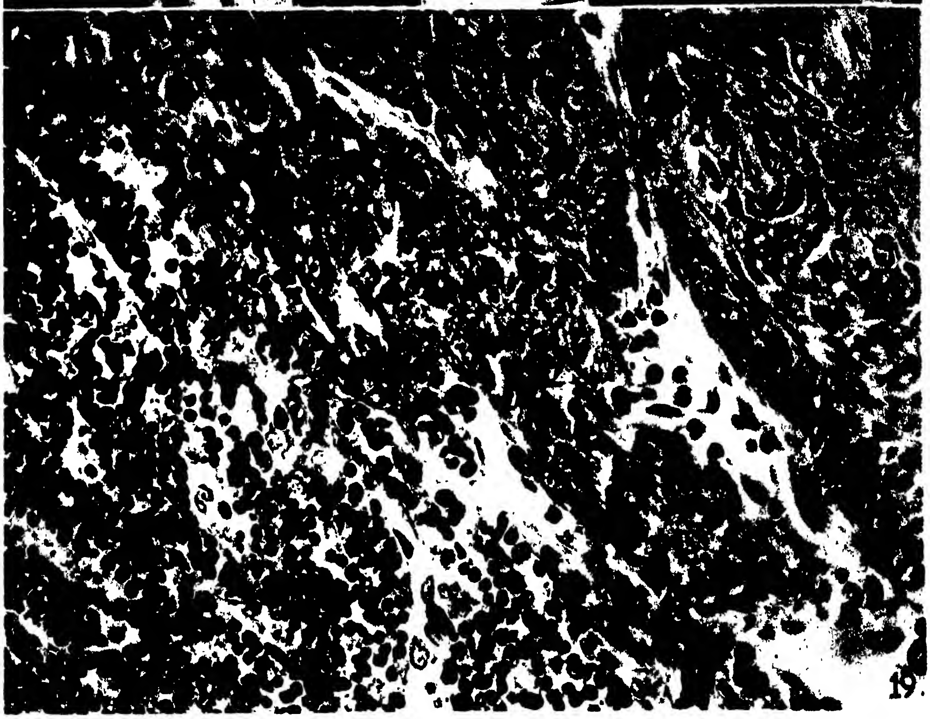
(Rous and Kidd: Carcinogenic effect of papilloma virus. I)

Plate 15

Figs. 16 and 17. The ears of D. R. 1-31 (Experiment 2) on the 47th day. The aural concavities are full of confluent, scabbed, fungating growth, and great distortion exists. On the outer side are scattered mounds and bulgings, some being deep lying, benign papillomas capped with dark keratin, while others are malignant (C,C) and in some cases ulcerated. Two biopsies (Cx) had been made at the ear margin. At Y is the diffuse thickening mentioned in the text (page 415), and at Cs the bulge caused by a deep lying, cystic, malignant growth (*vide* Fig. 22). $\times$ 2/5.

Fig. 18. (*a*) Longitudinal slice through an ear of Figs. 16 and 17. The diversified markings of the fungoid tissue indicate the variety of the growths. At several places (C,C) they have extended through the cartilage and formed mounds on its outside, especially near the ear tip. (*b*) Slice through several of the embedded, acorn-shaped papillomas on the outer side of the ear. 2 are medium and dark gray respectively, and all are vertically striated. $\times$ 4/5.

Fig. 19. Invasion by the glandular metastasis shown in Fig. 5. $\times$ 400.



Photographed by Joseph B. Haulenbeek and Louis Schmidt

(Rous and Kidd: Carcinogenic effect of papilloma virus. I)

Plate 16

Fig. 20. Extension of an anaplastic growth through a lacuna in the ear cartilage: biopsy on the 38th day, rabbit 1-31 (Experiment 2).

Fig. 21. Random section through the edge of an ear, at autopsy of the same animal. A highly anaplastic, ulcerating growth has extended through the cartilaginous plate. Nearby is a deep lying, heavily pigmented, acorn-shaped papilloma. × 10.

Fig. 22. Cross section of the bulging cyst, Cs, of Fig. 17. It is lined with malignant growth (Fig. 23), which can be seen also in a broad, ulcerated expanse opposite it on the inner side of the ear. × 4.

Fig. 23. Part of the cyst wall. × 40.

Photographed by Louis Schmidt

(Rous and Kidd: Carcinogenic effect of papilloma virus. I)

Plate 17

Fig. 24. Results of a profuse virus infection of skin tarred for many months previously (rabbit 6-51, Experiment 3: 23rd day after virus injection). Within the greatly thickened aural shells 2 large tar tumors can still be seen amidst much new-formed, fungating tissue. $\times$ 2/5.

Fig. 25. Same ears on the 28th day. They are distorted and rendered enormous by diffuse, papillomatous proliferation, which is almost entirely subepidermal on their outer side and along their edges. They weighed 220 gm. Nowhere did malignancy exist. $\times$ 2/5.

Figs. 26 and 27. Ears of rabbit 12 on the 29th and 42nd days: to show the rapid progress of the changes. $\times$ 2/5.

Fig. 28. Ulcerated, malignant growth on the outside of the ear of rabbit 27 (Experiment 4): 67th day. The bracket indicates its situation. The other growths are gray papillomas. Shadows complicate the picture. $\times$ 4/5.

Fig. 29. Extension to a salivary gland of the metastatic tumor at the base of the right ear of rabbit 28 (Experiment 4). $\times$ 42.

Fig. 30. Virus-induced, bulging, papillomatous excrescences on an old, pedunculated tar wart. Some of them are patched with gray. The surface of the wart had previously been smooth (D. R. 6-46, Experiment 3: 36th day). $\times$ 2/5.

Photographed by Joseph B. Haulenbeek and Louis Schmidt

(Rous and Kidd: Carcinogenic effect of papilloma virus. I)

PLATE 18

FIG. 31. Malignant growth on the inner surface of the right ear of rabbit 28: 67th day. It has encroached upon a nearby papilloma of dark hue. $\times$ 2/5.

FIG. 32. Extension of the growth of Fig. 31 to the outer surface of the ear: 107th day. $\times$ 2/5.

FIG. 33. Same growth on inner surface, with metastases in the auricular glands: 123rd day. (See also Fig. 14.) $\times$ 2/5.

FIG. 34. Destructive growths on the other ear of the rabbit, with glandular metastases: 123rd day. (See Fig. 14.) $\times$ 2/5.

FIG. 35. Left ear of Fig. 34 on the 156th day. Only an edematous, ulcerated stump remains, covered with dry blood. There is swelling round about and a large metastatic mass at the angle of the jaw. $\times$ 1/5.

FIG. 36. The ears and metastatic masses from behind: 156th day. $\times$ 1/5.

FIG. 37. Section through stump of the left ear: 159th day. $\times$ 11.

Photographed by Joseph B. Haulenbeek and Louis Schmidt

(Rous and Kidd: Carcinogenic effect of papilloma virus. I)

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THE COURSE OF VIRUS-INDUCED RABBIT PAPILLOMAS AS DETERMINED BY VIRUS, CELLS, AND HOST*

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PLATES 21 AND 22

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The lesions caused by viruses range in character from the acutely necrotizing to the continually proliferative. Little is known, however, of the relationship of these pathogenic agents to the cells they affect beyond the fact that they have what appears to be an obligatory association with the latter. The living cells, not the organism of which they are a part, function as the real hosts of a virus, protecting it from such antiviral principles as may circulate in the blood. In the light of these circumstances one can readily understand how recovery comes about from virus infections causing death of the cells, for with this occurrence protection ceases, and the virus is exposed to such antiviral forces as the host may have possessed or acquired during the disease. No such explanation will serve, however, to explain the observed retrogression of growths due to those viruses which cause the infected cells to multiply in series. Under such circumstances one might expect the pathological proliferation to go on inevitably until death of the animal ensued. This does not always happen, however. Virus-induced growths vary widely in their course and frequently they dwindle and disappear (chicken tumors, rabbit papillomas). The study reported here was carried out with a view to learning more of the respective parts played by a virus, the cells it acts upon, and the individual host in determining the course of a virus-induced growth. Special attention has been given to the phenomenon of retrogression. As material the rabbit papillomas caused by the Shope virus (1) were utilized, for the reason that they are sharply circum-

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scribed, autochthonous growths of neoplastic character (2), which can be directly measured and scrutinized; and because they are known to go on growing even though strong antiviral antibodies appear in quantity in the blood of rabbits bearing them (3).

Primary Influence of the Virus and of the Cellular Fabric upon Which It Acts

The early course of the growths produced by inoculation of the papilloma virus into scarified skin can be referred to the pathogenic activity and the concentration of the virus material engendering them, and to the character of the cellular fabric upon which this acts. The primary influence of the virus on the behavior of the papilloma can be likened to that of a shot fired into a medium of greater or less resistance. A highly pathogenic virus may cause papillomas to grow for a time in a relatively unfavorable milieu, just as a shot of great velocity may produce some effect on a resistant medium. A weak virus can give rise to progressive papillomas in highly susceptible hosts as a shot of low velocity can travel far if little resistance is offered. Some virus strains produce papillomas sooner than do others, and titration tests have shown that the incubation time is generally shortest (with an irreducible minimum of about 7 days) in the case of the strains of virus that prove effective in greatest dilution; as furthermore that these active strains produce more vigorous growths, when inoculated according to a standard technique, than do strains with longer incubation periods.

Papillomas due to one inoculum may run a highly various course, however, even in related domestic rabbits. In some individuals the virus causes fleshy, vigorous papillomas, while in others of the same age and weight it gives rise to dry-topped, shallow-based growths which retrogress. Certain differences in thickness, opacity, and succulence seem to mark the skins of some rabbits as more or less favorable to growth of the papilloma; but repeated attempts to ascertain the character of these differences by histological study, or by micrometric measurement of skin thickness during life, have thus far proved unsuccessful. Whatever the nature of the differences they are not specific, for comparative tests have shown that those skins in which

vigorous papillomatosis is engendered react with a more profuse epidermal proliferation to an intradermally injected solution of Scharlach R in olive oil, than do the skins which give rise to indolent or retrogressing papillomas. An illustrative experiment will be given.

Experiment 1.—6 adult, gray-brown, domestic rabbits weighing 2000 to 2500 gm. were utilized, in which tattoo papillomas from the same inoculum had run different courses. In 2 of the animals the papillomas were large and fleshy; they had enlarged rapidly and steadily during the 15 weeks since the virus inoculations. In 2 others small, shallow based, low, dry-topped growths had enlarged slowly; while in the remaining 2 the punctate papillomas had appeared somewhat late and enlarged very slowly, retrogressing completely 3 to 4 weeks after their appearance. The hair was clipped away from both sides of these rabbits and 0.1 cc. of a saturated solution of Scharlach R in olive oil was injected intradermally through a fine needle into 6 comparable sites on each side of every animal. The sites were reinjected with 0.1 cc. of the Scharlach R preparation 7 and 14 days later. When the results were appraised, 5 days after the last of the injections, notable differences were found. Reactive mounds had been produced in all of the animals, and in any one individual the mounds were almost identical in character. Those in the rabbits bearing fleshy papillomas consisted of large, fleshy, scarlet papules with sharp contours: they measured 10 to 16 mm. across and were elevated 6 to 9 mm. above the surrounding skin. In the rabbits in which the papillomas had retrogressed on the other hand, the mounds were lower and smaller (10 to 14 mm. across, elevated 3 to 7 mm.), much less fleshy, and they graded more gradually into the surrounding skin. In the 2 rabbits with indolent papillomas the mounds were intermediate as regards size, fleshiness, and color. Microscopic section of several of the various sorts, procured by operation on the 5th day after the last injection, bore out the gross findings. The injections had elicited a marked epidermal proliferation in the skin of the rabbits bearing fleshy papillomas (Fig. 1), whereas in the skin of those in which the growths had retrogressed the proliferation was far less marked (Fig. 2).

These findings have been borne out in several further experiments with a number of skin irritants (dibenzanthracene in olive oil and extracts of coal tar in olive oil, corn oil, cottonseed oil, cocoanut oil, palm oil, and paraffin oil) applied by injection and inunction. Generally speaking, skins which reacted markedly to one irritant did so to others, and in them the papilloma virus caused the most vigorous growths.

The Rôle of the Virus in the Phenomenon of Retrogression

In a first attempt to ascertain the rôle of the virus in the phenom-

enon of retrogression, a group of comparable domestic rabbits were inoculated at many separate situations with 3 strains of virus known to differ in primary pathogenicity.

Experiment 2.—10 normal adult gray-brown rabbits weighing between 2000 and 3350 gm were secured from a single dealer. All were inoculated at 4 points on each side with 3 different strains of virus. The first material (virus fluid A) was made from the naturally occurring warts of a cottontail rabbit trapped in East Texas.[1] These had been in 50 per cent glycerol for 6 weeks. Representative portions of the glycerolated warts were weighed, washed briefly in three changes of Tyrode's solution, and ground with sand. Sufficient Tyrode's solution was then added to make a 10 per cent extract, the extract was centrifugalized lightly, and the supernatant fluid was pipetted off for use. Two more 10 per cent extracts were prepared identically from material generously furnished by Dr. Shope,—virus fluid B from the natural warts of a Kansas cottontail (W.R. 1240), in glycerol for 8 months, and virus fluid C from those of a second Kansas cottontail (W.R. 1211), in glycerol for 10 months. The inoculations were made with an electric tattoo machine fitted with nine small sewing needles. A small drop of virus fluid was placed at each of several sites on the shaved skin and uniformly tattooed into an area about 2 mm. across. Immediately after the tattooing, the excess of virus was blotted up with a sterile sponge. 4 such inoculations were made with each virus fluid along horizontal lines on each side of the rabbits. The inocula were thus placed 3 to 4 cm. from one another, and their relative positions were systematically varied from rabbit to rabbit, so that all were exposed to the same influences of situation. A single virus fluid was inoculated into every rabbit before the next was proceeded to, and the tattoo needles were sterilized before each material was utilized.

The growths resulting from these inoculations were charted at intervals of 2 to 6 days. Chart 1 gives a summary of the findings. From this it will be seen that all 3 strains of virus caused papillomas in every inoculated rabbit, but that strain A was far less effectual than were strains B and C. Host susceptibility found general expression, the rabbits most susceptible to one virus strain being most susceptible to the others as well.

In 3 of the 10 rabbits (50, 51, 44) all the papillomas, of whatever derivation, enlarged steadily throughout the many weeks of observation; and it is noteworthy that all the growths on any one animal eventually attained a fairly uniform size, regardless of their virus derivation or differing primary course. In 6 animals, on the other hand, retrogression of all the growths took place simultaneously after a time; and the chart shows that in three of these cases (49, 47, 43) the enlargement had been as great during the early weeks as in the rabbits (50, 44) in which the papillomas grew progressively. In the case of D.R. 48, all of the growths began

[1] Secured through the endeavor of Mr. R. C. Adams.

CHART 1. Comparative course of the papillomas induced by 3 strains of virus (A, B, C) in 10 domestic rabbits (Experiment 2).

to dwindle between the 26th and 30th days, and those from one strain of virus,—that which had given rise primarily to the most rapidly enlarging growths,—actually disappeared for a while, only to recur later. Plainly, in this instance, some intercurrent, generalized resistance of host derivation transiently influenced the course of all of the papillomas, though to an unequal extent,—the most rapidly proliferating cells being evidently most susceptible to its action. This phenomenon of transient intercurrent retrogression will be discussed more fully in a later section.

In this experiment retrogression of the papilloma was not due to peculiarities of the virus strains employed, but was consequent on some generalized influence exerted by the host; for, as a rule, all of the growths on any one animal enlarged or retrogressed together, even when engendered by virus strains of widely various pathogenicity.

The Recovery of Virus from Retrogressing Papillomas

What can be the nature of the generalized host influence responsible for retrogression of the papillomas in instances such as the foregoing? Mention has already been made of the fact that the sera of rabbits bearing the papilloma develop the capacity to neutralize free virus *in vitro* or on reinoculation. Previous experiments have demonstrated that the circulating, virus-neutralizing antibodies do not perceptibly influence the course of the growths in domestic rabbits, papillomas enlarging as rapidly in such rabbits when the blood has great antiviral potency as when this is slight (3). The living papilloma cells evidently protect the virus from the action of humoral antiviral principles, as other cells do other viruses (4). Because of this protection it should be possible to recover active virus from retrogressing papillomas, if it has not become attenuated in some other way.

In the attempt to recover the virus from retrogressing papillomas recourse was had to wild cottontail rabbits, for from experimentally induced growths in these natural hosts active virus can be procured frequently, instead of rarely as in domestic rabbits. The gross and microscopic manifestations of retrogression are similar in the two species, as many instances have shown. Furthermore, in the cottontail retrogression of the papilloma is the result of a generalized process, just as in the domestic species, and multiple growths induced by differ-

ent strains of the virus retrogress simultaneously. The following experiment demonstrates that active virus can be recovered from retrogressing papillomas.

Experiment 3.—10 "normal" cottontail rabbits from Kansas were inoculated broadcast on both scarified flanks with a highly active virus fluid (5 per cent suspension of the glycerolated papillomas of W.R. 1211), prepared as previously described. Growths appeared after about 10 to 15 days, but in 8 animals they were small, discrete, low, dry warts, and in several they soon retrogressed. Exuberant growths appeared, however, in 2 rabbits. One of these was killed on the 28th day and its growths preserved in glycerol; the other (W.R. 27-N) was ob-

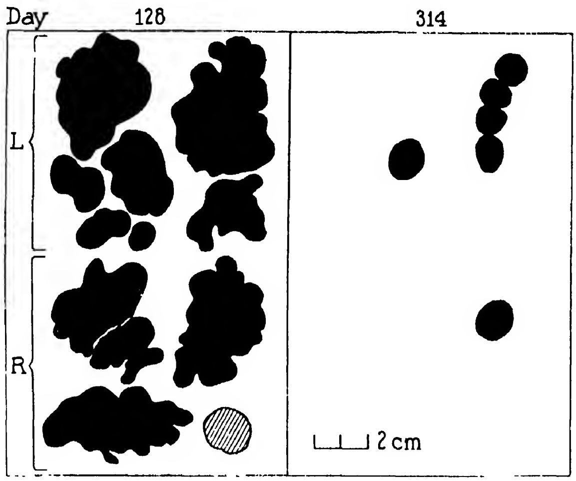

CHART 2. Retrogression and disappearance of the papillomas of W.R. 27-N (Experiment 3).

The hatched area indicates the outline of a subepidermal cyst near the superficial areas of confluent papillomatosis, such as is often seen about growths of exceptional vigor in cottontails. Virus was recovered in large amount from the small, dwindling papillomas present on the 314th day.

served over a long period. Its growths enlarged rapidly and progressively during the first 4 months to form characteristic, large, confluent and semiconfluent, papillomatous masses, 3 to 5 cm. across and 1 to 2 cm. high, with cystic extensions into the connective tissue beneath and around them, of the sort forming when cottontail growths have exceptional vigor (5). During the subsequent months, nevertheless, they dwindled notably and most of them disappeared, as did all of the subcutâneous cysts. Tracings made on the 128th and 314th days, which illustrate the changes, are given in Chart 2. On the latter day, when the animal was killed, there remained on the left side 5 dry, pigmented, shallow, onion-shaped papillomas with constricted bases, none over 1.3 cm. in diameter; and a single, similar growth on the right side. These 6 small growths were excised

aseptically and put into sterile 50 per cent glycerol-Locke's solution in the refrigerator.

10 months later part of the glycerolated material was weighed, washed briefly in Tyrode's solution, ground with sand and made up to a 5 per cent suspension in Tyrode, and tested for infectiousness, along with 20 other suspensions made from glycerolated papillomas from as many cottontails in which growth had been progressive. All were inoculated into squares of skin on each of 3 normal domestic rabbits, according to a standard technique (3). By the 15th day thereafter, multiple, discrete growths had appeared on 2 of the rabbits where the W.R. 27-N suspension had been inoculated, and by the 19th day on the third rabbit as well. The growths due to this virus enlarged steadily into wholly characteristic, confluent and semiconfluent, fleshy, papillomatous masses, and they showed no tendency to retrogress. Upon comparison with the results from the 20 other materials it was evident that the W.R 27-N virus was notably pathogenic, the growths caused by it being even more vigorous than were those caused by most of the inocula which had been derived from enlarging papillomas. A second 5 per cent virus fluid prepared a few weeks later from portions of the glycerolated W.R. 27-N papillomas again produced large, characteristic growths when inoculated into 3 new test rabbits. The serum of W.R. 27 N, which had been secured by cardiac puncture on the day the papillomas were obtained, was found to neutralize completely a 5 per cent suspension of highly active virus (W.R. 1240,—more than 2000 infective units per dose) when mixed with it in equal parts *in vitro* and incubated at 37°C. for 2 hours prior to inoculation.

A later experiment yielded similar results. Another retrogressing papilloma from a second cottontail (W.R. 21-N) had become available for test, -namely a small, discrete, dry growth 0.8 cm. in diameter, secured on the 243rd day after inoculation. On the 116th day the papilloma had been 2.5 cm. in diameter, fleshy, truncated and onion-shaped, and it had undergone the reduction to its later size during the intervening period, while a number of other large papillomas produced on the animal by the same inoculum were retrogressing completely. A 5 per cent extract in Tyrode derived from it produced semiconfluent and discrete papillomas by the 15th day after inoculation in 2 of 4 domestic rabbits, and by the 20th day confluent and semiconfluent growths in all.

It is plain from these instances that the virus can persist in a highly pathogenic state in papillomas that have long been retrogressing, even though virus-neutralizing antibodies of high potency are circulating in the blood of the host. The implications of these facts will be discussed in a later section.

The Rôle of the Proliferating Cells

The evidence thus far given makes plain the fact that retrogression

of the rabbit papilloma is consequent on some general influence of host derivation, but it does not suffice to disclose the nature of this influence. Certainly retrogression can take place despite the continued presence of active virus in association with papilloma cells. This being so, one can scarcely suppose the process to be due to the antiviral forces of the host. Indeed everything indicates that the cells completely protect the virus against these forces.

The possibility suggests itself that the virus-infected cells may on occasion call forth a host resistance directed against themselves. To test this possibility an experiment was done in which the amount of papillomatous tissue was greatly varied in 3 groups of animals, and the incidence and course of retrogression were followed.

Experiment 4.—30 normal, adult, gray-brown domestic rabbits were secured from a single dealer, weighed, and notations made on the texture and thickness of their skins. The animals were then divided into 3 groups of 10, each group comparable in weight and skin characteristics. One group received on each side 32 identical tattoo inoculations of 10 per cent W.R. 1240 virus; the second group received 12 such inoculations, while the last group received only 2 on each side. At each site the tattooing was done over an area about 2 mm. across until a slight amount of blood came out into the drop of virus, which was then blotted off with a sterile sponge. The inocula were placed at points at least 2 cm. from one another.

As in the previous experiment all of the rabbits proved primarily susceptible to the action of the virus, growths appearing at almost every inoculation site, and nowhere else. There was considerable variation from host to host, but the growths on any one animal were of the same order of magnitude and fairly uniform as regards fleshiness and pigmentation. They were charted at intervals of 2 to 7 days until the 53rd day, and at longer intervals thereafter. The findings are summarized in Charts 3, 4, and 5. One of the animals died 3 days after the virus inoculation and hence is not represented in the charts. The early findings showed the 3 groups to be comparable as regards primary host susceptibility, and the incidence of retrogression did not vary significantly in the 3 groups.

Chart 3 shows the growths in 10 animals from the 3 groups in which the papillomas grew progressively, and in an eleventh in which a dubious late dwindling occurred. The findings are given for comparison with those next to be considered, and they need but little discussion. It will be observed that the papillomas showed a slight tendency to become larger eventually in the animals bearing fewer growths; but differences in the blood supply available to each growth, when there were 2, 12, ot 32 on a side, respectively, may account for this difference, and crowding may have had some influence when many growths were present.

Chart 4 shows the course of events in 13 animals of the 3 groups, in which complete retrogression eventually took place. While the incidence of retrogres-

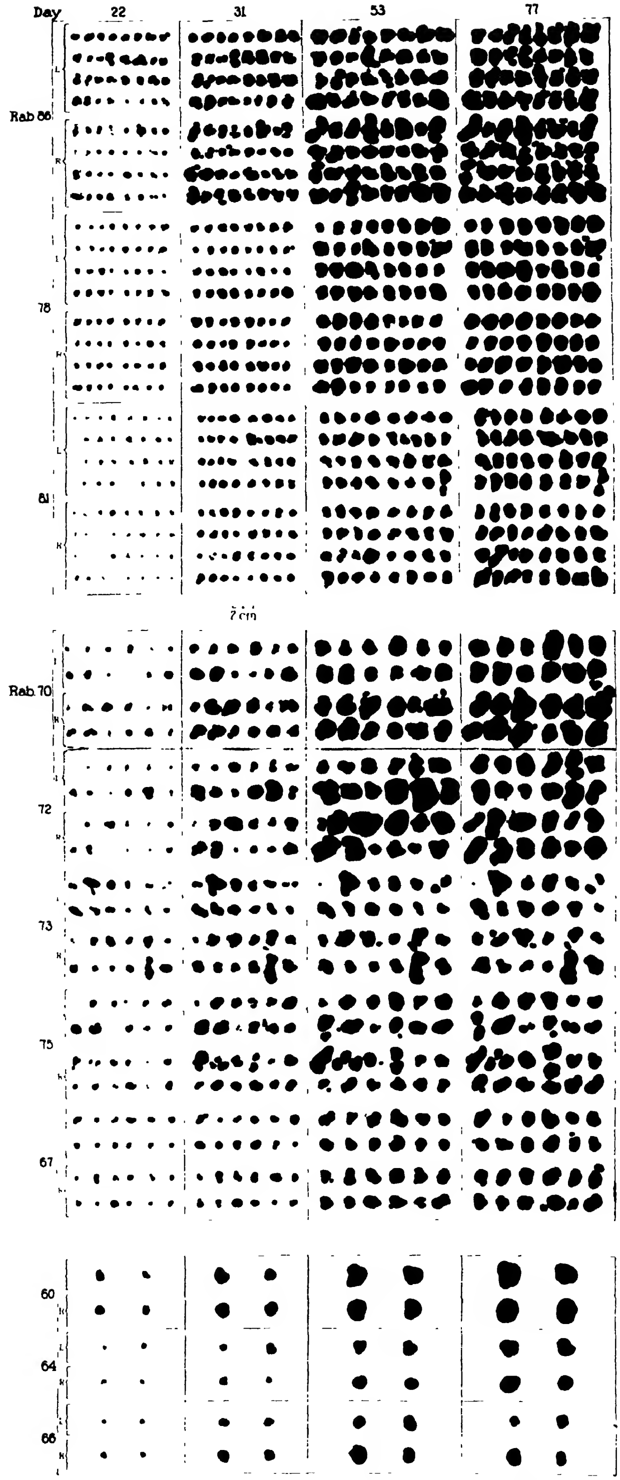

CHART 3. The progressing papillomas of 11 rabbits in Experiment 4. L, R = left and right sides respectively.

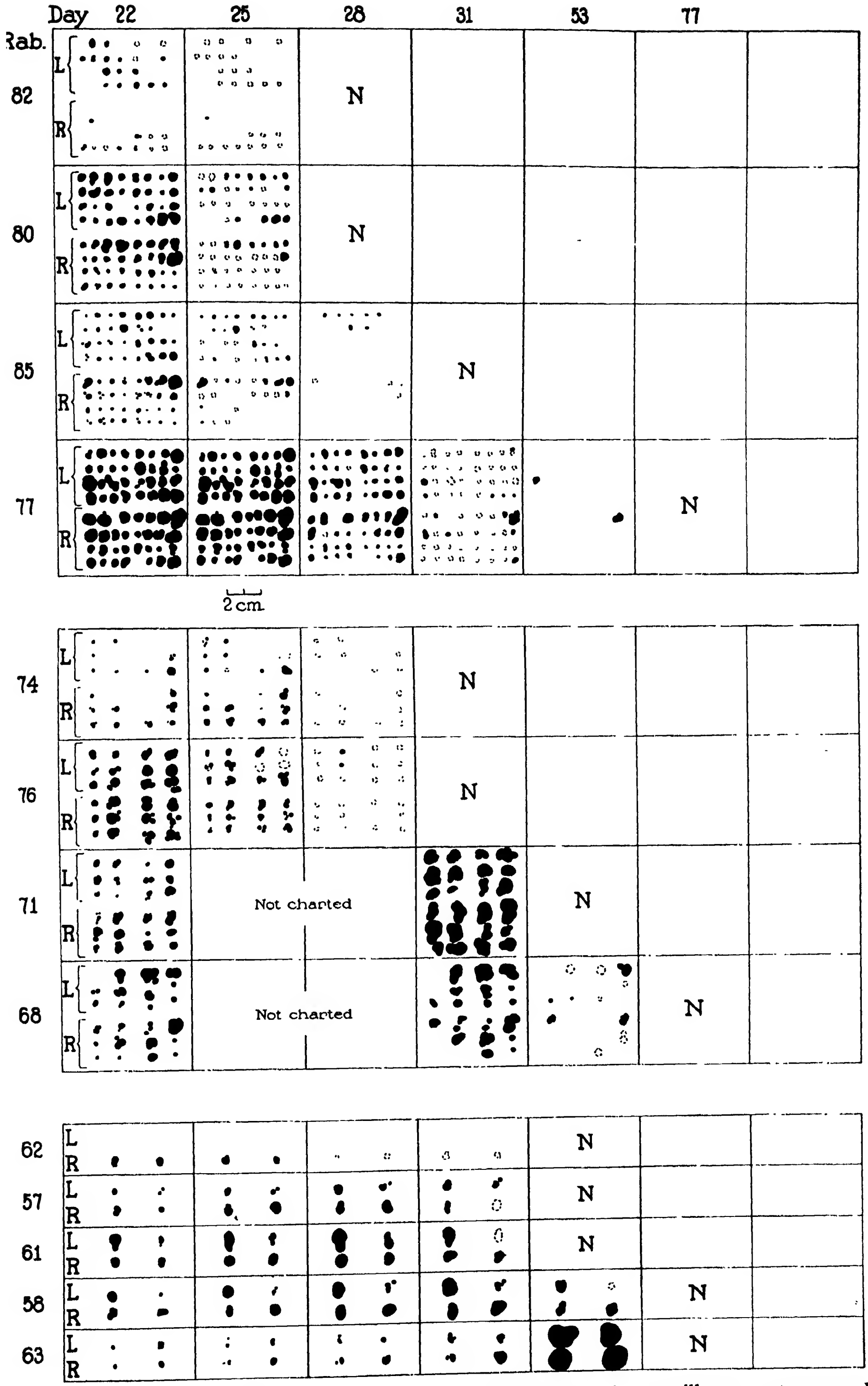

CHART 4. In the rabbits bearing many small growths the papillomas retrogressed earlier and more swiftly than in the others with few (Experiment 4). L, R = left and right sides respectively.

sion did not vary significantly from group to group, its time of onset and course did so markedly, as the chart shows. It will be seen that retrogression tended to begin sooner, and to take place more swiftly, the larger the number of papillomas present on a host, although the individual growths did not differ notably in size. In the 4 animals with 64 papillomas retrogression began earlier and was com-

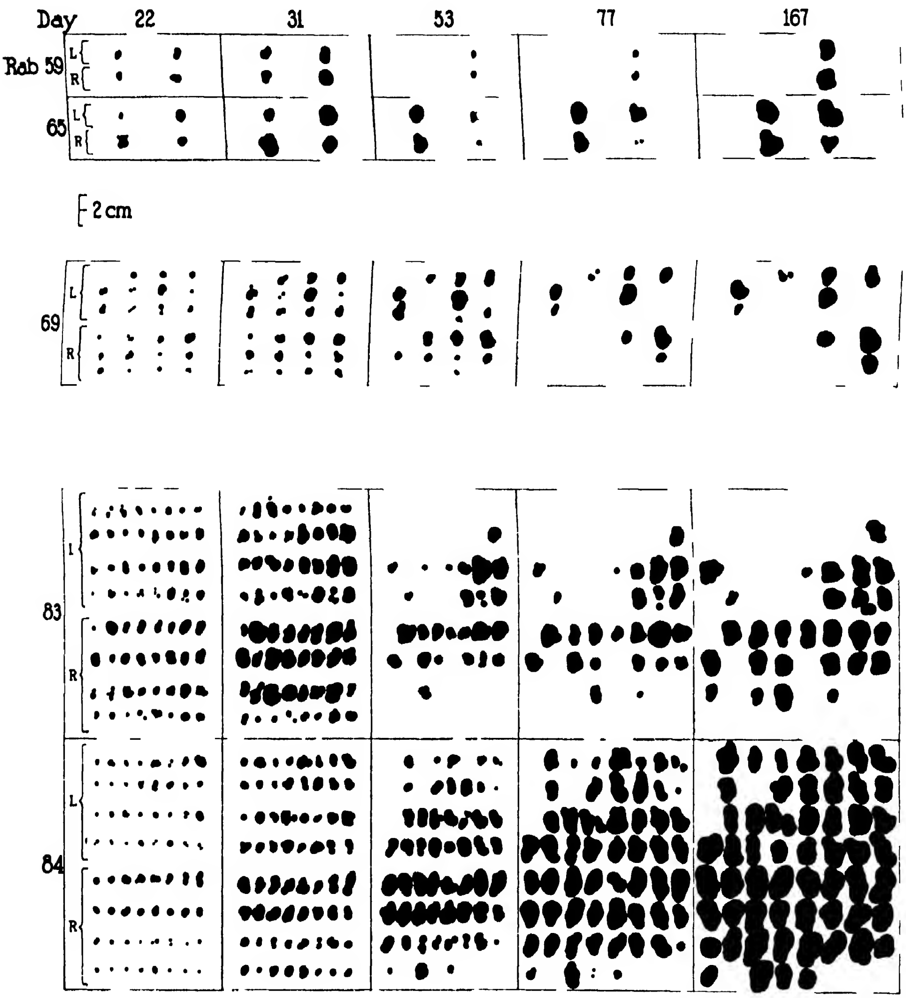

CHART 5. Transient, intercurrent retrogression of the papillomas in 5 domestic rabbits of Experiment 4. L, R = left and right sides respectively.

pleted more abruptly than in the animals bearing 24 papillomas. In these, in turn, retrogression took place earlier and more swiftly than in the rabbits with only 4 papillomas.

In the remaining 5 animals of the experiment a transient, intercurrent tendency to retrogression was evident. The findings (Chart 5) are comparable with those

in rabbit 48 of Chart 1. In one or two animals of each group some of the papillomas (usually the smaller) disappeared completely and permanently between the 31st and 53rd days, while concurrently other growths either disappeared transiently, dwindled somewhat, remained the same size, or, in rare instances, enlarged slightly. When all of the persisting papillomas began to grow again, some, which had disappeared leaving mere small, dry, shallow, scurfy thickenings in their places, reappeared as characteristic papillomas and enlarged steadily like the rest.

The influence of some generalized host resistance acting to bring about retrogression is clearly to be seen in the results of this experiment, as in Experiment 2 already discussed. In the present instance it was manifest in about the same number of individuals of each group, but to an unequal degree. Often the resistance was only transiently and feebly effective. The greater the number of papillomas induced, which is to say, the greater the total amount of proliferating epithelial tissue, the sooner did retrogression occur in the hosts predisposed thereto, and the more rapidly was the process consummated. Since the influence of virus-induced antibodies can be ruled out as responsible for the course of events, there is reason to suppose that the host resistance was directed against the cells themselves; and there is the more ground for thinking so because the manifestation of this resistance varied directly, both in time of appearance and in effectiveness, with the amount of papilloma tissue present.

The Histological Changes during Retrogression

The histological findings where papillomas are retrogressing constitute significant evidence of a host resistance directed against the proliferating cells as such, for they are essentially identical with those observed where transplanted tissues (neoplastic or non-neoplastic) retrogress as a consequence of resistance known to be directed against the cells.

The microscopic changes associated with retrogression of the rabbit papilloma have already been described, and the resemblance noted to those about retrogressing tissues generally (2). In a further study of the matter, sections of a number of small, discrete, retrogressing growths stained with eosin and methylene blue have been examined. The results extend the previous findings.

Much of the retrogression appears to be due to a slower rate of cellular proliferation, with result that differentiation outstrips it and death of the cells occurs through maturation into keratinized scales. It is often possible to find areas where

this process is more advanced than elsewhere; and in these the cells are stained less darkly, and mitoses are less frequently seen, while lymphocytes and makrophages are often present in considerable number in the underlying connective tissue. Everywhere the living papillae become progressively thinner, the epithelial layer more shallow, and finally there is left only a smooth scar covered by epidermis somewhat thicker than the normal, but appearing to be merely hyperplastic. The whole process suggests a gradual waning of cellular activity under the influences of an unfavorable environment, with eventual death by maturation of all the remaining virus-infected cells.

Attempts to Elicit Resistance Directed against the Papilloma Cells

In view of the evidence indicating that retrogression of rabbit papillomas may be due to a generalized host resistance elicited by and directed against the papilloma cells as such, it seemed well to make a direct attempt to bring about such resistance experimentally. In the case of transplantable mouse and rat tumors this has been accomplished by preliminary injections of living cells (embryo skin, blood, etc.) into animals to which the growths were to be transplanted. The animals were thus rendered more or less refractory, like hosts in which the tumors had previously retrogressed. Acting upon this knowledge, considerable quantities of rabbit embryo skin were repeatedly injected into one group of rabbits; the Brown-Pearce epithelioma was transplanted to another group to obtain individuals in which spontaneous retrogression of this tumor had taken place; and a third, comparable group to which nothing was done were retained as controls. All were finally inoculated with the papilloma virus, and the resulting growths were observed and their outlines charted from time to time.

Experiment 5.—7 adult gray-brown rabbits of 2000 to 2500 gm. were injected with minced rabbit embryo skin. Three courses of injection were carried out at intervals of 4 to 5 weeks, the last injections being given 2 weeks before the experiment was begun. Each course consisted of 4 subcutaneous injections of 1 or 2 cc. of a heavy suspension of freshly procured and hashed 23 to 26 day rabbit embryo skin. A second group of 7 similar rabbits were kept untreated but under the same conditions, and when the experiment was done 7 more rabbits of the same breed and from the same dealer were utilized, in which good sized Brown-Pearce tumors in the skin, subcutaneous tissues, and muscles had retrogressed 2 to 3 months previously.

The 21 rabbits were inoculated identically by tattooing active 5 per cent virus (Texas strain) into four spots about 2 mm. across on each side. After 15 to 25 days small papillomas appeared at all of the inoculation sites. The developing

growths were charted at intervals of 5 to 7 days until the 11th week after inoculation, when the experiment was discontinued.

There was no discernible difference in the behavior of the growths in any of the 3 groups, either as regards incidence, time of incubation, size, rate of enlargement, or onset or incidence of retrogression. In about half the animals in each group the papillomas grew progressively; in the remainder they did poorly, either retrogressing abruptly after 6 to 7 weeks or dwindling more slowly after 7 to 11 weeks.

The negative outcome of this experiment was not unexpected, since the growths induced by the virus consisted of the animal's own cells. Similar attempts by others to alter the course of established transplanted growths, or of autochthonous tumors arising spontaneously, or as a result of tarring have also failed (6). This fact, and the experiments just reported, illustrate the limitations of artificial immunization with unaltered homologous tissues. These elicit at best a relatively feeble immunological response, one that can hardly be expected to prevail against "spontaneous" tumors or established transplanted growths, nor even against the papilloma, a growth of exceptional vigor, which can be produced by inoculation of the virus in all normal domestic rabbits, irrespective of whether they will constitute favorable hosts later on.

The Influence of Local Conditions

Local conditions often play a part in determining the fate of the papilloma, and they not infrequently influence the phenomenon of retrogression. Small, discrete growths retrogress much more readily than do large, crowded, confluent papillomatous masses on the same animal, a fact which can be best perceived when the virus is inoculated into many individuals by methods which yield large and small growths respectively.

On many occasions Shope virus has been rubbed into broad areas (about 10 x 12 cm.) of scarified skin on the abdomens of normal gray-brown domestic rabbits with result that large, confluent papillomatous growths occupying most of the inoculated areas appeared after 8 to 16 days. When an active virus material had been employed the confluent growths of this sort generally enlarged into enormous masses, and they did not often retrogress. When this did happen, the process began only after months, and then the tumors often dwindled so slowly that more months were required before they disappeared altogether. It was repeatedly noted that they dried down and flaked away slowly from the periphery inwards;

and often an area of papillomatosis which had become confluent secondarily by the apposition of masses growing from separate foci was broken up by the process of retrogression into its component parts, each of the latter then dwindling from its edges toward its center.

When the same virus fluids were inoculated with the tattoo machine at many widely separated points (about 2 mm. in diameter) or rubbed into several small, scarified rectangles (about 3 x 4 cm. in dimension) on the abdomens and flanks of comparable hosts, the later course of events was very different. The growths first appeared as small, rounded papillomas at the tattooed sites, or confluent or semiconfluent, small growths on the scarified rectangles. These all enlarged rapidly. Often in a third or more of the inoculated animals, 3 to 6 weeks after the virus inoculations, the papillomas, then up to 8 mm. in height, abruptly ceased to enlarge and began to dry and dwindle away, and within a few days all had disappeared. In contrast with the group already described, retrogression began soon and was swiftly concluded.

These observations make it plain that retrogression of the rabbit papilloma, like that of growths consequent on implantation (7), takes place predominantly from the periphery inwards; while in confluent growths where large numbers of primarily affected cells are crowded closely together, retrogression is in some way forestalled or hindered. The slow rate at which large growths retrogress has some connection perhaps with the relatively small ratio of periphery to total size.

So pronounced is the response of the papilloma to such local growth-promoting influences as partial excision, local inflammation due to bacteria, or injections of Scharlach R in olive oil (2), as to suggest the possibility that intercurrent local factors may cause the papilloma to grow progressively at some situations, while at others lacking their influence, it would retrogress. To test this possibility multiple, discrete papillomas were produced on the sides of rabbits, and those on one side were stimulated repeatedly while their fellows on the opposite side served as controls.

Experiments 6, 7, and 8.—Three experiments were done, with 14 normal, adult, gray-brown rabbits. Virus was tattooed into the skin at 6 or 8 small spots on each side. 4 to 5 weeks later, when the papillomas had reached a diameter of 3 to 8 mm. and were up to 5 mm. high, those on the left side of each animal were injected with 0.1 cc. of a saturated solution of Scharlach R in olive oil. The dye was put into the base of the growths through a fine, hypodermic needle inserted

at one edge. The injections were repeated at weekly intervals until 5 to 9 had been given.

In 6 of the 14 animals the control papillomas enlarged progressively, as did the injected ones, the latter far more swiftly. The course of events in one of these animals illustrates clearly the extraordinary growth-promoting influence of the Scharlach R injections. Figs. 3 and 4 show the growths of this animal on the 183rd day after virus injection. 8 injections of 0.1 cc. of Scharlach R in olive oil had been made into the papillomas on the left side between the 26th and 75th days, those on the right being left undisturbed. On the 61st day one growth on each side was removed from corresponding situations for histological study, but these later recurred. When the photographs were taken the undisturbed growths were ordinary, small, discrete papillomas consisting mostly of keratinized peaks deeply cleft and dry almost to their bases; while the injected growths had become

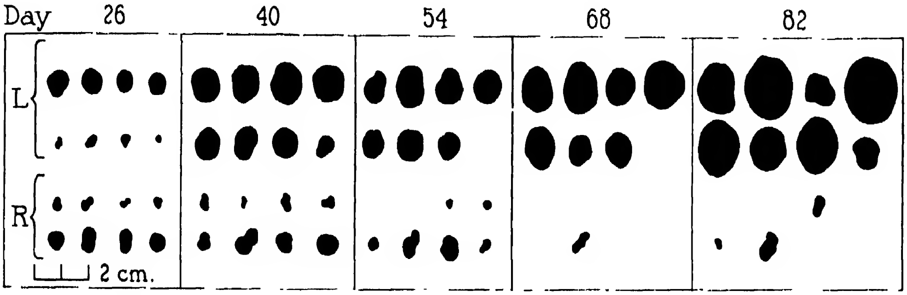

CHART 6. The early course of the growths of D.R. 21 (Experiment 8). The papillomas on the left side (L) were repeatedly injected with Scharlach R in olive oil; those on the right (R) were left undisturbed. For the later course of events see Figs. 5, 6, and 7.

huge, coalescent, fleshy discs, studded with innumerable "pearls." Their surfaces were ulcerating and weeping, and practically devoid of keratinized material, with large, rounded, subepidermal protrusions extending laterally from their bases in many places.

In 7 of the 14 animals the injected and control growths retrogressed simultaneously 4 to 6 weeks after the virus inoculations, but in two instances they disappeared rather slowly and the papillomas that had been injected, and in consequence had reached a larger size, required 7 to 14 days longer to disappear completely than did the control growths on the same animals.

Striking differences in the course of the injected and control growths were observed in the remaining rabbit (D.R. 21, Figs. 5, 6, and 7). In this animal vigorous, discrete papillomas had appeared at all of the inoculated sites. Those on the left side were injected with 0.1 cc. of a saturated solution of Scharlach R in

olive oil on the 26th, 33rd, 40th, 47th, 54th, and 61st days after the virus inoculations, whereas those on the right were not interfered with. Their course during the first 82 days is shown in Chart 6. It will be seen that the control papillomas on the right side of the animal became smaller and disappeared between the 40th and 68th days, leaving flat, smooth, pigmented scars,—except for one, which remained as a small, dry, scab-like wart. During this period the injected growths stopped enlarging, some dwindled slightly, and the smallest became obscured by the reactive proliferation called forth by the Scharlach R injections. Between the 68th and 82nd days this growth reappeared, however, and later it grew to a considerable size. While it was doing so, growths reappeared on the right side at two situations, and the one already present enlarged a little. Photographs (Figs. 5 and 6) taken on the 91st day show the stimulated growths as 8 large, fungoid papillomatous masses, and those on the control side as 2 small, scab-like warts (w) with 3 flat, pigmented scars (s) marking the situations of papillomas that had disappeared. By the 121st day the surface growths on the injected side were dwindling rapidly; underneath them at this time, however, small, rounded, smooth, subcutaneous nodules (pearls) had appeared, and a biopsy of one of them on this day revealed an epidermal cyst 0.8 cm. in diameter, filled with lamellated, keratinized scales and surrounded by a somewhat irregular rind of differentiating papilloma tissue up to ½ mm. thick, embedded in dense reactive tissue consisting largely of fibroblasts. 3 weeks later the surface growths on the injected side had disappeared entirely, as had also the small, dry scabs from the control side. At this time subcutaneous cysts, 0.2 to 2.0 cm. in diameter, were to be seen at 6 sites on the left side, pushing up the scars that marked sites whence the stimulated surface papillomas had retrogressed. These cysts persisted for many months, often rupturing spontaneously through the skin to discharge yellowish, dry, lamellated material. 2 of them received 4 injections of 0.1 cc. of Scharlach R in olive oil during the 8th month, but they were not notably changed thereby. Fig. 7, taken on the 280th day, shows the cysts then present at 5 sites. The rabbit was killed 13 months after the virus inoculation and 9 months after the last of the surface growths had disappeared. Although no stimulating injections of Scharlach R had been given for 4 months prior to the animal's death, several of the cysts persisted. At autopsy, 3 of them were present 1 to 3 cm. in diameter, and one had ruptured through the skin and was discharging lamellated material. The microscope showed that all were simple cysts filled with keratinized scales and lined with a living, proliferating layer of papillomatous epithelium encapsulated in sclerotic connective tissue.

Retrogression occurred in 6 rabbits in these experiments. In 3 it involved the papillomas injected with Scharlach R and the control growths simultaneously, whereas in 2 others the injected papillomas persisted for a few days after the control growths had disappeared

completely. In the sixth instance, however, the injections caused the papilloma cells to proliferate for many months after the control growths on the same animal had retrogressed entirely. In this case the resistance developed by the host against the papillomas was of but moderate degree, and under these circumstances the Scharlach R injections proved the determining element in their fate.

It is plain that favorable local conditions can influence notably and even decisively the fate of the papilloma. Occasionally they may account for the persistence of growths which might otherwise be overcome by a retrogressive influence of generalized character. Such exceptional instances appear analogous to those previously cited of transient intercurrent retrogression, in which only the smaller of a number of discrete growths disappeared, the larger persisting, though much diminished in size, and eventually growing again. Evidently in some instances the balance is finely drawn between the proliferative activity of the enlarging papillomas on the one hand, and the resisting host forces on the other. When this is the case, local conditions may prove decisive for the fate of the growths.

DISCUSSION

Virus-induced tumors are the result of a singular partnership, with cells and virus working actively together to produce a disease. When conditions are favorable to both, vigorous growths arise; but such conditions do not always obtain. The experiments here reported show that conditions sometimes become bad for the cells of the rabbit papilloma, although remaining good for the virus associated intimately with them; and in these instances the growths retrogress, despite the sustained pathogenicity of the virus.

Retrogression of the rabbit papilloma is the result of a generalized resistance of host origin, elicited by the proliferating cells (Experiment 4); but this resistance is distinct from that directed against the virus itself, as manifested in the development of humoral, antiviral antibodies. These antibodies neutralize the virus *in vitro*, yet they do not influence the course of the growths it induces. In some rabbits the growths enlarge progressively although nourished by blood of high antiviral potency, whereas in others in which the antiviral titer

of the serum is comparatively low, they dwindle and disappear (3). The living papilloma cells evidently protect the papilloma virus, as other cells do other viruses (4), and apparently they protect it amply. If humoral antibodies ever neutralized the virus associated with papilloma cells not otherwise damaged, one might expect these to revert to the normal; but no evidence of any such occurrence has been encountered in the many sections of retrogressing growths examined with this point in view. The papilloma cells remain such until they die.

In what way can retrogression of the papillomas take place despite the continued presence of highly pathogenic virus in association with them? The histological changes about retrogressing papillomas are identical with those taking place where other proliferating tissues (neoplastic or non-neoplastic) retrogress in consequence of resistance directed against the cells. Although the precise character of the host reaction responsible for retrogression is still uncertain,[2] it is known, in the case of transplanted tissues, to be the expression of resistance elicited by and directed against the proliferating cells (10).

During retrogression some transplanted tumors grow smaller by disappearance of their peripheral tissue, and the multiple growths of any one animal retrogress simultaneously (7). Both these phenomena are to be observed in the case of the papilloma. The host influence responsible for the retrogression of transplanted growths is sometimes transient in its effects, with result in alternate enlargement and dwindling (10), and this is true of the papilloma as well. When host resistance is weak neither transplanted growths nor the virus-induced papillomas need adjuvant influences to enable them to grow, and when it is strong none of the influences thus far tested can prevent their retrogression; but when it is indecisive inter-

[2] Woglom (8) has reported the recovery and partial purification of an agent from the spleens of rats rendered resistant to a transplanted tumor which will act against tumor cells *in vitro*; and the recent experiments of Gorer (9) provide evidence that the resistance effective against transplanted tumor cells, at least in certain instances, is the result of an immunological response elicited because of antigenic differences between the tissues of the animal in which the growth arose, and those of its new host.

current factors influencing for good or ill the proliferation of the growths may determine their fate, as the present work and previous experience have shown (10). From this it follows that retrogression of the papilloma, though affecting the animal's own cells, is similar in its manifestations to the retrogression of transplanted tumors; and good reason exists for supposing that it is brought about by the same means, namely by a host resistance directed against the cells.

Lacassagne has recently found that retrogression of the rabbit papilloma can be brought about with Roentgen rays in dosages which suffice to kill the papilloma cells *in situ*, but do not harm the virus *in vitro* (11). The retrogression due to the rays, like that which is the outcome of natural forces, as described in the present paper, must be referred to conditions that are bad for the cells but ineffective against the virus associated with them. Woglom has pointed out (10) that during retrogression of transplanted tumors cell division often still goes on in some parts of the growth, as if they were still in good state. It is doubtless in such regions, which have also been noted in retrogressing papillomas, that the virus persists in active form.

Individuals do not ordinarily manifest resistance directed against their own tissues, but this phenomenon is not unknown. Certain viruses cause alterations in the constituents of cells which they infect, with result that these become autogenous antigens and elicit immunological responses (12); and resistance manifested by an individual against its own tissues has been encountered in other states (autohemagglutination, paroxysmal hemoglobinuria), either as a natural occurrence or induced experimentally (13).

Factors similar to those influencing the course of the rabbit papilloma can be discerned in the case of the virus-induced tumors of fowls. The vigor of these growths varies both with the pathogenicity of the virus inoculum, and with the susceptibility of the cellular fabric upon which it acts (young and old hosts, different breeds). In some fowls conditions are favorable both to the virus and to the cells that become infected with it, and in these individuals the growths are largest. In others, less fortunate conditions obtain and retro-

gressing growths are got, the histological changes then indicating that the retrogressive influence is directed against the cells. The cells of retrogressing fowl tumors do not revert to the normal, but *remain such until they die, just as happens in the case of the papilloma.* The fowl tumor cells protect the causative viruses from the action of humoral antiviral principles, as do the papilloma cells (14). These various phenomena illustrating the respective rôles of virus, cells, and host provide a close parallel with the state of affairs in the virus-induced papillomas of rabbits. It seems probable that the same general principles will be found to hold good in the case of other tumors due to viruses.

SUMMARY

An experimental analysis of the factors responsible for the observed differences in the course of virus-induced papillomas of the rabbit has shown that some are referable to the virus, others to the cells, and yet others to host influences. The interplay of these factors affords enlightening illustration of the nature of the cell-virus relationship in virus-induced tumors. Retrogression of the rabbit papillomas appears to be consequent on a generalized resistance of host origin, elicited by and directed against the proliferating, virus-infected cells.

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EXPLANATION OF PLATES

PLATE 21

FIGS. 1 and 2. To illustrate individual differences in the epidermal response to proliferative influences. The specimens were procured from 2 rabbits 5 days after the last of 3 intradermal injections of Scharlach R into a skin site previously normal. Such repeated injections had been made at 8 spots in each animal, and the specimens photographed are representative. One of the rabbits carried large and vigorous virus-induced papillomas, whereas in the other, inoculated at the same time and with the same material, the growths had retrogressed after brief proliferation. It will be seen that the epidermis from the individual with vigorous papillomas (Fig. 1) exhibits far the greater hyperplasia. ×8.

FIGS. 3 and 4. To show the effect of injections of Scharlach R in olive oil on enlarging papillomas. Fig. 3 shows the ordinary, discrete papillomas existing on one side of a rabbit 183 days after virus had been tattooed into the skin at 8 spots. × ¾. Fig. 4 shows growths of the same origin on the opposite side of the animal, which had received 8 injections each of Scharlach R in olive oil at weekly intervals between the 26th and 75th days. The undisturbed growths are mere, small papillomas, consisting mostly of keratinized cleft peaks, dry almost to their bases, while the injected growths have become huge, coalescent, fleshy discs with ulcerating surfaces and many nodular, subepidermal extensions from their bases. × ¾.

Plate 22

Figs. 5, 6, and 7. To show the influence of injections of Scharlach R in olive oil on the papillomas of a rabbit with moderate resistance to the growths. Fig. 5 shows growths which had become large, fungoid, papillomatous discs in consequence of injections of the dye (D. R. 21—91st day). × 3/5. Fig. 6 shows the uninjected growths on the opposite side of the animal on the same day. 2 small, dry scab-like warts (w) alone remain of the 8 original growths. The sites of 3 others are marked by flat, pigmented scars (s). × 3/5. The scurfy areas near the centers of Figs. 5 and 6 mark the sites where the Scharlach R preparation had been injected into normal skin. Fig. 7 shows subepidermal, papillomatous cysts persisting at several situations where the superficial papillomas of Fig. 5 had long since retrogressed, as had the growths on the opposite side. 280th day. × 3/5.


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EXPERIMENTAL SYPHILIS OF ORIENTAL ORIGIN: CLINICAL REACTION IN THE RABBIT

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For many years it has been reported that the clinical manifestations of syphilis in tropical and oriental regions differ in several important respects from the disease usually seen in occidental countries. The most striking and frequently mentioned differences are a low incidence of general paresis and tabes, and the frequently severe character of secondary manifestations. Although the majority of reports lack laboratory evidence, the clinical observations are impressive, especially with respect to the comparative infrequency of parenchymatous neurosyphilis. Several suggestions have been advanced to account for this so called exotic syphilis. Among the first of these was the strain theory, that is, that strains of *Treponema pallidum* differ in their disease producing properties. Special or peculiar qualities of reaction of different races have also been invoked, and lately consideration has been given to the influence of recently introduced treatment and the presence of endemic malaria. It has also been suggested that, if, as some have thought, involvement of the central nervous system is a relatively late feature in the biological history of syphilis in the western world, one would not expect its incidence to be as high in communities in which the disease has recently been introduced as in those in which it has been present for many centuries.

The question of differences of syphilitic reaction as indicated by such terms as occidental, oriental, exotic and tropical syphilis has a larger significance than is ordinarily implied by such designations. An integral part of the subject concerns the essential nature of the tropical disease yaws or frambesia which some observers contend is in reality a form of syphilis. The precise status of another disease, "bejel," described as the endemic syphilis of the Euphrates Arab is still unde-

termined (1). This disease, which is non-venereally acquired, has little effect upon the general constitution, its lesions are principally mucocutaneous, cutaneous and periosteal, while the nervous and cardiovascular systems and the viscera escape serious damage. Syphilis in rural populations of southeastern Europe is also said to be endemic and relatively mild (2).

It has not been possible until comparatively recently to study the subject experimentally in the laboratory, but our knowledge of the biology of experimental syphilis in the rabbit is now sufficiently comprehensive to permit the investigation of certain features of the matter. To this end a study of two oriental strains of *T. pallidum* of Chinese origin was undertaken first in Peiping and subsequently in New York on the basis of the character of the reaction to infection in the rabbit as compared with two well known occidental strains of American origin. The results of these comparative experiments are reported in the present paper.

A special feature of the investigation concerned the animals employed. To avoid the complications which might be introduced by a variable animal material, rabbits of both American and Chinese sources were used in all experiments. The American rabbits were shipped from New York to Peiping shortly before the Peiping experiments and similarly Chinese rabbits were sent to New York. It is of interest to note that the animals stood the long voyage *via* the Panama Canal and the Yellow Sea, transhipment in Japan, and a rail journey in China remarkably well.

Materials and Methods

The study comprised four experiments, two in Peiping in 1931–32 and two in New York in 1932-33. Four strains of *T. pallidum* were employed, two of oriental and two of occidental origin. The oriental strains of *T. pallidum* employed, designated Chinese III and IV respectively, had been isolated 5 and 3 years previously from patients in the hospital of the Peiping Union Medical College. Both strains were known to produce well marked clinical manifestations in the rabbit. Chinese III strain was obtained by the intratesticular inoculation of rabbits of cerebrospinal fluid from a patient with an acute meningitis and a primary chancre. Dark field examination of the centrifuged fluid showed actively motile spirochetes. The strain was in the 12th generation of rabbit passage at the beginning of these experiments. Chinese IV strain was similarly obtained from an inguinal lymph node of a patient with secondary syphilis. There were multiple bone lesions and a

marked general glandular enlargement. The strain was in its 11th rabbit passage. The American strains employed were the well known Nichols and Zinsser-Hopkins organisms, which have been used for many years in the majority of studies on experimental syphilis in the United States. In both Peiping experiments all four strains were used. In the first New York experiment the Nichols and the Chinese III strains were used and in the second New York experiment the Nichols and the Chinese IV strains.

For source material the western strains were always carried in American and the eastern in Chinese rabbits. The emulsion for inoculation was prepared from the tissue of an acute orchitis with sterile salt solution. Dark field examination of the suspensions showed from 2 to 6 actively motile organisms per microscopic field. The dose employed was 0.2 cc. injected intratesticularly or intracutaneously.

The net total number of rabbits inoculated was 320 (Table I). Observations made on animals which died during the experiments are not included. The great majority of rabbits, namely 262, were inoculated in one testicle; 106 were injected with the Nichols, 25 with the Zinsser-Hopkins, 65 with the Chinese III and 66 with the Chinese IV strains respectively. A supplementary group in which the intradermal route was used was included in the second Peiping experiment (Table I). 58 rabbits were inoculated in the skin of the sheath, 13 rabbits with each of the American strains and 16 rabbits with each of the Chinese strains.

The rabbits were adult males derived from American and Chinese stocks. The American rabbits were hybrids with Dutch, English and Lilac crosses predominating; the Chinese rabbits were Albino mixtures and many were rather small delicate animals. Comparable numbers of animals from both sources were represented in each experiment. The intratesticular experiments in Peiping comprised 50 American and 51 Chinese rabbits and those in New York 72 American and 89 Chinese rabbits. For the intradermal experiment 28 American and 30 Chinese animals were used.

The rabbits for each set of experiments were assembled at the same time. When inoculated, those of the first Peiping and New York experiments were 6 to 7 months of age while those of the second Peiping and New York experiments were 8 to 9 months old. Each rabbit was caged separately from the time of assemblage throughout the periods of shipment and experiment. The diet in Peiping consisted of soy beans, cabbage and hay, and in New York of commercial food pellets, oats, hay and a free supply of water. These diets were also used during the two voyages.

The basis of comparison of the strains was the character of the clinical reaction to infection, and detailed clinical examinations were carried out at least three times a week. Special attention was paid to the following: (*a*) the frequency and time of occurrence of the primary orchitis; (*b*) the frequency and time of occurrence of critical edema in the inoculated testicle; (*c*) the frequency and time of occurrence of lesions in the uninoculated testicle (metastatic orchitis); (*d*) the frequency, time of occurrence and number of generalized lesions in the bones and periosteum, the

TABLE I

Experimental Data

Strain of *T. pallidum*	Experiment	Number and source of rabbits		
		Total	American	Chinese
Intratesticular inoculation				
Nichols	Peiping 1	10	5	5
	"	16	7	9
	New York 1	40	20	20
	" " 2	40	17	23
	Total....	106	49	57
Zinsser-Hopkins	Peiping 1	9	5	4
	" 2	16	8	8
	Total....	25	13	12
Chinese III	Peiping 1	9	5	4
	" 2	16	7	9
	New York 1	40	20	20
	Total....	65	32	33
Chinese IV	Peiping 1	9	5	4
	" 2	16	8	8
	New York 2	41	18	23
	Total....	66	31	35
	Total.............	262	125	137
Intracutaneous inoculation				
Nichols	Peiping 2	13	5	8
Zinsser-Hopkins	" "	13	7	6
Chinese III	" "	16	8	8
Chinese IV	" "	16	8	8
	Total.............	58	28	30
Total............	....	320	153	167

skin and mucous membranes, and the eye; (*e*) the proportion of animals that showed complete healing of all lesions at the termination of the experiments. The period of observation in the Peiping experiments was 4 months and in the New York experiments 3½ months.

In the analysis of the results group means have been employed, a procedure which tends to minimize the effect of the chance occurrence of severe syphilis in any one group. In the case of generalized lesions, the numbers given represent the numbers of discrete foci at which lesions developed as determined by actual count. The figures for actual rates or actual distribution represent the mean values for those animals of the group in which generalized lesions actually developed while the relative rates indicate the results in terms of the entire group.

RESULTS

The results of the experiments are recorded in Tables II to VII. The four principal experiments, two in Peiping and two in New York, in all of which the intratesticular route of inoculation was employed will first be discussed, and then follows the supplementary experiment in which the intradermal route of inoculation was used. The particular features of the infection selected for discussion are the outstanding clinical manifestations of the experimental disease.

Primary Orchitis.—Every rabbit of the 262 inoculated intratesticularly developed a primary orchitis (Table II). The mean incubation periods of the lesion were 18.5 and 14.5 days respectively for the Nichols and Zinsser-Hopkins strains and 18.9 and 15.8 days for the Chinese III and IV strains (Table III). It will be noted that for each strain there was comparatively little variation of the mean values of individual experiments from the mean group value. The greatest variation occurred with the Chinese III strain, the incubation period of the three experiments being 21.0, 18.4 and 17.3 days respectively.

Critical Edema.—Edema of the scrotum and tunics of the inoculated testicle is a less constant feature than other phenomena of infection and variability of results is to be expected. Its mean incidence in these experiments was as follows: Nichols strain 53.8 per cent; Zinsser-Hopkins strain 34.7 per cent; Chinese III strain 36.5 per cent, and Chinese IV strain 52.8 per cent (Table II). The values for the Nichols and the Chinese IV groups do not include the results of the second New York experiment because at the time when edema was appearing in these rabbits, rabbit pox (3) had developed in the colony. In certain instances it was thought that the edema was more probably related to pox than to the syphilitic process, and consequently the figures relating to this feature were omitted. There was considerable variation in edema incidence in the individual experiments. Considering only the two Peiping experiments the results with both Chinese strains were less variable than those with the American strains. In the first New York experiment the value for the Nichols strain returned to the level of the first Peiping experiment, while that for the Chinese III strain was also increased and exceeded considerably both Peiping figures.

The mean times of development of critical edema were generally comparable, that is, 27.5 and 24.4 days after inoculation for the occidental strains and 26.9

and 22.8 days for the oriental strains respectively (Table III). There was a slightly greater variation in the individual experimental values from their group means in the case of the Chinese strains, that is, 2 days more or less than the group mean as compared with 1 day for the American strains.

TABLE II

Incidence of Various Phenomena of Infection and Focal Distribution of Generalized Lesions

Strain	Experiment	Number of rabbits	Primary orchitis	Critical edema	Metastatic orchitis	Distribution of generalized lesions: Incidence	Focal distribution: Actual	Focal distribution: Relative
			per cent	*per cent*	*per cent*	*per cent*		
Nichols	Peiping 1	10	100.0	70.0	90.0	100.0	5.6	5.6
	" 2	16	100.0	31.3	81.3	87.5	5.3	4.7
	New York 1	40	100.0	60.0	75.0	70.0	5.5	3.9
	" " 2	40	100.0	*	92.0	75.0	9.2	6.9
	Total.........	106	100.0	53.8	84.8	83.1	6.4	5.3
Zinsser-Hopkins	Peiping 1	9	100.0	44.4	77.8	100.0	5.9	5.9
	" 2	16	100.0	25.0	100.0	93.8	4.8	4.5
	Total.........	25	100.0	34.7	88.9	96.9	5.4	5.2
Chinese III	Peiping 1	9	100.0	33.3	66.7	88.9	5.6	5.0
	" 2	16	100.0	31.3	75.0	87.6	4.4	3.8
	New York 1	40	100.0	45.0	72.5	80.0	7.0	5.6
	Total.........	65	100.0	36.5	71.4	85.5	5.7	4.8
Chinese IV	Peiping 1	9	100.0	55.6	88.9	100.0	6.3	6.3
	" 2	16	100.0	50.0	80.0	87.5	4.9	4.3
	New York 2	41	100.0	*	80.4	68.3	5.6	3.9
	Total.........	66	100.0	52.8	83.1	80.9	5.6	4.8

* Omitted because of intercurrent pox infection.

Metastatic Orchitis.—Clinical involvement of the uninoculated testicle was a frequent feature of the disease produced by all four strains (Table II). The mean incidence with the Nichols, the Zinsser-Hopkins and the Chinese IV strains was comparable, that is, 84.8, 88.9 and 83.1 per cent respectively; it was lower with the Chinese III strain, 71.4 per cent. There was comparatively little variation

in the values for individual experiments with the Chinese strains; for the four experiments with the Nichols strain the incidence ranged from 75.0 to 92.5 per cent and for the two Zinsser-Hopkins experiments, the values were 77.8 and 100.0 per cent.

TABLE III

Mean Time of Occurrence of Various Phenomena of Infection Estimated from the Date of Inoculation

Strain	Experiment	Number of rabbits	Primary orchitis	Critical edema	Metastatic orchitis	Generalized lesions			
						First lesion	Mean of all	Last lesion	Duration of active period
			days	*days*	*days*	*days*	*days*	*days*	*days*
Nichols	Peiping 1	10	19.4	28.6	54.1	57.9	66.8	74.1	20.4
	" 2	16	18.9	26.6	53.7	60.3	71.1	88.4	28.1
	New York 1	40	18.5	27.3	52.0	52.2	57.8	66.9	15.2
	" " 2	40	17.1	*	50.9	58.2	65.5	74.8	13.7
	Total.........	106	18.5	27.5	52.4	57.2	65.3	76.1	19.1
Zinsser-Hopkins	Peiping 1	9	14.6	25.2	69.3	56.9	68.0	73.1	19.0
	" 2	16	14.3	23.5	44.2	64.8	72.3	92.8	29.0
	Total.........	25	14.5	24.4	56.8	60.9	70.2	83.0	24.0
Chinese III	Peiping 1	9	21.0	29.0	56.5	59.0	67.2	78.5	20.0
	" 2	16	18.4	26.6	53.0	57.1	60.8	71.1	17.1
	New York 1	40	17.3	25.1	47.4	43.7	47.1	57.7	16.4
	Total.........	65	18.9	26.9	52.3	53.3	58.4	69.1	17.8
Chinese IV	Peiping 1	9	16.7	24.8	48.9	51.6	60.5	71.8	20.8
	" 2	16	15.5	20.8	44.6	54.8	59.8	70.7	20.2
	New York 2	41	15.5	*	46.5	49.6	54.0	63.1	15.2
	Total.........	66	15.8	22.8	46.7	52.0	58.1	68.5	18.7

* Omitted because of intercurrent pox infection.

The mean time at which metastatic orchitis was first detected was 52.4, 56.8 and 52.3 days after inoculation for the Nichols, Zinsser-Hopkins and Chinese III strains respectively; it was definitely earlier, 46.7 days, for the Chinese IV strain (Table III). For the four individual experiments with the Nichols strain and the three with the Chinese IV strain, the values were quite uniform, the range of variation being only 2 days from the group mean. For the three experiments

with the Chinese III strain, however, this variation amounted to 5 days and with the two Zinsser-Hopkins experiments 13 days.

Generalized Lesions.—The most significant single index of pathogenicity of a strain of *T. pallidum* is the production of generalized lesions in remote parts of the body. This phase of the disease comprises lesions of the periosteum and bone, the skin and mucous membranes, and the eyes. Involvement of the scrotum and tunics is not usually included since these may result from the direct extension of the primary lesion.

The animal incidence of generalized lesions in these experiments was high and remarkably uniform, the mean values being 84.8 and 96.9 per cent respectively for the Nichols and Zinsser-Hopkins strains and 85.5 and 80.9 per cent for the Chinese III and IV strains (Table II). Considering only the Peiping experiments, the mean results were 93.8 and 96.9 per cent for the two American strains and 88.2 and 93.5 per cent for the Chinese III and IV strains. The values in New York were consistently lower than in Peiping. For the two Nichols groups the mean incidence was 72.5 per cent; for the single Chinese III and IV groups the values were 80.0 and 68.3 per cent respectively. The results for the two Zinsser-Hopkins and the three Chinese III experiments were quite uniform; for the four Nichols groups the incidence ranged from 70.0 to 100.0 per cent and for the three Chinese IV experiments from 68.3 to 100.0 per cent.

The focal distribution of generalized lesions was next considered. The mean values given in Table II show that the results for the four strains were of the same general order. The highest values for both actual and relative rates occurred with the Nichols strain, namely, 6.4 and 5.3 respectively, but the figures for the other strains are but slightly lower, that is, Zinsser-Hopkins 5.4 and 5.2; Chinese III 5.7 and 4.8; and Chinese IV 5.6 and 4.8. The grade of infection as judged by the values for individual groups was most severe with the Nichols strain in the second New York experiment; the values for the three other experiments with this strain were lower but very uniform. A similar uniformity is seen in the results of the experiments with the Zinsser-Hopkins strain and of the three with the Chinese IV strain. In the case of the three experiments with the Chinese III strain, the values were more irregular and the New York figures were slightly higher than those in Peiping.

The location of generalized lesions has been used as a further basis of comparison of the four strains of organisms (Table IV). With the Nichols strain the mean values and those of individual experiments as well showed a higher proportion of skin than bone lesions; with the Zinsser-Hopkins strain this relationship was reversed. The mean value for eye lesions was the same, 2.8 per cent, for both American strains. With the Chinese III strain, the mean values resembled those with the Hopkins strain as regards the bone-skin representation of lesions; and in two of the three individual experiments the results were of the same order. The proportion of eye lesions, however, was high, the mean value being 5.3 per cent. In the case of the Chinese IV strain the mean values for bone and skin lesions approximated those of the Nichols strain, that is, a larger proportion of skin le-

sions. In two of the three experiments, however, these values were almost identical. There was likewise a high proportion of eye lesions with the Chinese IV strain, the mean value being 6.8 per cent. Eye lesions developed in all three experiments with the Chinese IV strain, but they were not observed in one each of the experiments with the other strains.

The mean time of appearance of the first generalized lesion was slightly earlier with both Chinese than with the American strains, the mean values being 53.3 and 52.0 days after inoculation for the Chinese III and IV strains and 57.2 and

TABLE IV

Location of Generalized Lesions

Strain	Experiment	Total number	Bone		Skin		Eyes	
			No.	*per cent*	*No.*	*per cent*	*No.*	*per cent*
Nichols	Peiping 1	56	20	35.7	33	58.9	3	5.4
	" 2	75	32	42.7	40	53.3	3	4.0
	New York 1	154	65	42.2	89	57.8	0	—
	" " 2	276	34	12.3	238	86.2	4	1.5
	Mean........		38	33.2	100	64.0	3	2.8
Zinsser-Hopkins	Peiping 1	53	30	56.6	23	43.4	0	—
	" 2	72	36	50.0	32	44.4	4	5.6
	Mean........		33	53.3	28	43.9	2	2.8
Chinese III	Peiping 1	45	25	55.6	15	33.3	5	11.1
	" 2	61	26	42.6	32	52.5	3	4.9
	New York 1	224	127	56.7	97	43.3	0	—
	Mean........		59	51.6	48	43.1	3	5.3
Chinese IV	Peiping 1	57	27	47.4	26	45.6	4	7.0
	" 2	69	32	46.4	31	44.9	6	8.7
	New York 2	156	55	35.3	95	60.9	6	3.8
	Mean........		38	43.0	51	50.5	5	6.5

60.9 days for the Nichols and the Zinsser-Hopkins strains (Table III). The values for individual experiments were in general agreement with the mean group values with the exception of the Chinese III strain, the figures for which were 59.0, 57.1 and 43.7 days respectively. The mean time of appearance of the last generalized lesion was likewise earlier in the case of both Chinese strains, being 69.1 and 68.5 days for the Chinese III and IV strains and 76.5 and 83.0 days for the Nichols and Zinsser-Hopkins strains (Table III). A similar result occurred with respect to the mean time of development of all generalized lesions. For both these

phenomena, the results of individual experiments were in fair accord with their group means except in the case of the Chinese III strain which again showed considerable variation. The interval between the appearance of the first and the last generalized lesion has been selected to represent the period of generalized disease activity. The duration of this period was slightly but definitely shorter for both Chinese strains, the mean group values being 17.8 and 18.7 days for the Chinese III and IV strains and 19.1 and 24.0 days for the Nichols and the Zinsser-Hopkins strains. The mean duration periods of individual experiments showed more uniformity for the Chinese than the American strains.

Recovery.—At the end of each experiment all animals were classified on the basis of complete resolution and healing of all lesions (Table V). The observation period of the Peiping experiments was 4 months and of those in New York 3½ months. The proportion of completely negative animals was much higher with the Chinese than the American strains in the Peiping experiments, the mean values being 85.4 and 64.6 per cent for the Chinese III and IV strains as compared with 10.0 and 21.2 per cent for the Nichols and Zinsser-Hopkins strains. In the first New York experiment none of the rabbits inoculated with the Nichols strain had completely recovered as contrasted with 8.1 per cent recoveries among the rabbits infected with the Chinese III strain. This latter value, however, might be raised to 21.6 per cent by the inclusion of 5 rabbits which were "almost or practically negative." By this term is meant a slight fibrous residual thickening at the site of a previous lesion, and in the great majority of cases the condition eventually disappears. In the second New York experiment with the Nichols and the Chinese III strain, the proportion of complete recoveries was the same for both strains, 17.5 per cent. If a similar inclusion of 5 and 8 practically negative rabbits be made, the resulting values are 25.0 per cent for the Nichols and 36.6 per cent for the Chinese IV strain. It seems clear, therefore, that the clinical manifestations were definitely more enduring in the infections produced by the Nichols and Zinsser-Hopkins strains of *pallidum* than by the two Chinese strains employed.

Intracutaneous Inoculation.—The second Peiping experiment included 58 rabbits inoculated intracutaneously, 13 with each of the American strains and 16 with each of the Chinese strains.[1] The injections were made in the skin of the sheath with the same tissue emulsions used for intratesticular inoculation.

A primary chancre developed in each rabbit (Table VI). The mean incubation periods for the Nichols and the Zinsser-Hopkins groups were 23.3 and 14.8 days and for the Chinese III and IV groups, 20.4 and 17.4 days respectively. In general

[1] 16 rabbits were inoculated with each strain, but there were 3 early accidental deaths in the groups inoculated with the Nichols and the Zinsser-Hopkins strains.

the chancres produced by both western strains were larger, more indurated and more destructive than those of the Chinese strains; 75.0 per-cent of the chancres in the former groups were classified as large or medium sized as compared with 50.0 per cent in the latter groups. Metastatic orchitis developed in approxi-

TABLE V

Number and Percentage of Animals with Complete Healing of All Lesions at Termination of Observation Period. Peiping Experiments 4 Months; New York Experiments 3½ Months

Strain	Experiment	Number of rabbits	Complete healing of all lesions	
			No.	*per cent*
Nichols	Peiping 1	10	2	20.0
	" 2	16	0	—
	Total............	26		10.0
	New York 1	35*	0	—
	" " 2	40	7	17.5
Zinsser-Hopkins	Peiping 1	9	1	11.1
	" 2	16	5	31.3
	Total............	25		21.2
Chinese III	Peiping 1	9	7	77.1
	' 2	16	15	93.7
	Total............	25		85.4
	New York 1	37†	3	8.1
Chinese IV	Peiping 1	9	6	66.7
	" 2	16	10	62.5
	Total............	25		64.6
	New York 2	40‡	7	17.5

Final observations on 9 rabbits which died during the last fortnight of the experiments are not included in this summary: *5 rabbits; †3 rabbits; and ‡1 rabbit.

mately one-third of the rabbits inoculated with the Nichols and the two Chinese strains, but there was a higher incidence, 53.8 per cent, in the Zinsser-Hopkins group (Table VI). The mean incubation period of the orchitis was practically the same, 76 and 78.8 days for the Chinese strains; it was shorter, 67 days, for the Nichols strain, but for the Zinsser-Hopkins strain it was prolonged to 92.9 days.

As was to be expected with this route of inoculation, the animal incidence of generalized lesions was lower and their number smaller than was found with the intratesticular route. For the Nichols and Zinsser-Hopkins strains, the mean incidence was 38.5 and 84.6 per cent and for the Chinese strains III and IV, 75.0

TABLE VI

Incidence and Mean Time of Occurrence of Various Phenomena of Infection and Focal Distribution of Generalized Lesions. Intracutaneous Inoculation

Strain	Number of rabbits	Primary chancre		Metastatic orchitis		Generalized lesions		
		Incidence	Incubation period	Incidence	Incubation period	Incidence	Focal distribution	
							Actual	Relative
		per cent	*days*	*per cent*	*days*	*per cent*		
Nichols	13	100.0	23.3	30.7	67.0	38.5	3.5	1.3
Zinsser-Hopkins	13	100.0	14.8	53.8	92.9	84.6	3.0	2.5
Chinese III	16	100.0	20.4	37.5	76.0	75.0	2.5	1.9
Chinese IV	16	100.0	17.4	31.5	78.8	56.3	3.8	2.1

TABLE VII

Location and Mean Time of Occurrence of Generalized Lesions from the Date of Inoculation. Incidence of Complete Healing of All Lesions at Termination of 4 Months Observation Period. Intracutaneous Inoculation

Strain	Number of rabbits	Generalized lesions									Duration of generalized lesion activity	Animal incidence of complete healing of all lesions	
		Total number	Bone		Skin		Eyes		First lesion	Last lesion			
			No.	*per cent*	*No.*	*per cent*	*No.*	*per cent*	*days*	*days*	*days*	*No.*	*per cent*
Nichols	13	17	3	17.6	14	82.4	0	—	81.8	96.0	24.5	3	23.1
Zinsser-Hopkins	13	33	7	21.2	25	75.8	1	3.0	88.7	103.9	32.5	4	30.8
Chinese III	16	30	13	43.3	16	53.3	1	3.3	60.5	79.7	23.6	12	75.0
Chinese IV	16	34	6	17.6	24	70.6	4	11.7	84.9	97.7	18.6	12	75.0

and 56.3 per cent (Table VI). The focal distribution rates of the four groups were comparable, the actual rates being 3.5 and 3.0 for the American strains and 2.5 and 3.8 for the Chinese III and IV strains respectively.

The mean time of appearance of the first generalized lesion was approximately the same for the two American and the Chinese IV strains, the values being 81.8,

88.7 and 84.9 days after inoculation; for the Chinese III strain, however, it was considerably shorter, that is, 60.5 days (Table VII). A similar result occurred with respect to the appearance of the last lesions, that is, 96.0, 103.9 and 97.7 days for the two American and the Chinese IV strains and 79.7 days for the Chinese III strain. The mean duration of generalized disease activity for the American strains was 24.5 and 32.5 days; it was slightly shorter, 23.6 days, for the Chinese III strain and considerably shorter, 18.6 days, for the Chinese IV strain.

As is the rule with the intracutaneous route of inoculation, there was a predominance of skin and mucous membrane lesions (Table VII). The proportion of these lesions was 82.4, 75.8 and 70.6 per cent for the Nichols, Zinsser-Hopkins and Chinese IV strains respectively; a lower value, 53.3 per cent, occurred with the Chinese III strain. Involvement of the eyes was frequent with the Chinese IV strain, the proportion of lesions being 11.7 per cent as compared with 3.0 and 3.3 per cent with the Zinsser-Hopkins and Chinese III strains. No lesions of the eyes developed in the rabbits inoculated with the Nichols strain.

At the conclusion of the experiment, 4 months after inoculation, it was found that the incidence of rabbits in which all lesions had completely regressed and were healed was much higher in the animals inoculated with the Chinese strains. The actual values were 75.0 per cent for each Chinese strain and 23.1 and 30.8 per cent for the Nichols and Zinsser-Hopkins strains respectively (Table VII).

DISCUSSION

Before discussing the results of these experiments, one matter affecting the interpretation of the New York experiments should be referred to. In both these experiments there was an intercurrent rabbit pox infection and the question arises whether the findings can properly be compared with those previously obtained in Peiping.

The fact is well recognized that the manifestations of two concomitant infections may not be of the same order as those of each infection when present alone. It has been shown, moreover, that, under certain conditions and particularly those associated with the relative times of inoculation, the manifestations of experimental syphilis of the rabbit are affected by a concomitant vaccinal infection (4). In the present instance the outbreak of pox occurred toward the end of the first New York experiment, that is, at the beginning of the 4th month after inoculation. The experiment was at once terminated and no lesion not known to have been present before pox was detected was included in the results. The second New York experiment was well under way before the pox outbreak. It occurred just prior to the development of the syphilitic critical edema. Since scrotal edema also occurs in pox, the observations on this feature of the results were omitted from present consideration. That the intercurrent infection had some effect upon the subsequent course of the syphilitic condition is not improb-

able. For example, the comparatively short duration of generalized disease activity in this experiment (Table III) may be an instance in point. It will be noted, however, that the findings of all experiments have been presented separately and comparison of the tabulated values shows that the majority of results of the second New York experiment did not disagree in any major particular with those obtained in the previous experiments. It will also be noted that the evaluation of the final condition of the animals with respect to the proportion of rabbits which showed complete resolution and healing of lesions was based primarily on the longer and uncomplicated Peiping experiments.

The results of the four experiments here reported show first, that the general grade of infection produced in the rabbit by two strains of *T. pallidum* of Chinese origin was quite similar to that produced by two occidental strains, the Nichols and the Zinsser-Hopkins. There were no noteworthy differences in the incidence of primary and metastatic lesions nor in the severity of the generalized phase of the disease as judged by the focal distribution rate of generalized lesions. In pathogenicity both Chinese strains were comparable to the high level of the American strains. This result recalls the similar experience of Hu (5) with another and recently isolated Chinese strain, the pathogenicity of which was likewise pronounced from the first rabbit passage.

Although the oriental strains did not appear to evoke any quantitative peculiarities of reaction, there were certain qualitative differences between the disease produced by them and the American strains. The most evident difference concerned involvement of the eyes. Of the total number of generalized manifestations the mean proportion of eye lesions was 2.8 per cent for each American strain as compared with 5.3 and 6.5 per cent for the Chinese III and IV strains respectively (Table IV).

In the longer and uncomplicated Peiping experiments the animal incidence of eye lesions was 2.8 per cent for each Chinese strain and 2.3 and 0.8 per cent for the Nichols and Zinsser-Hopkins strains. The incidence of eye lesions in terms of their total possible number was: Chinese III strain 16.0 per cent; Chinese IV strain 16.0 per cent; Nichols strain 11.5 per cent; and Zinsser-Hopkins strain 8.0 per cent. Finally, of the generalized lesions, the mean proportionate values for eye lesions were 4.7 and 2.8 per cent for the Nichols and Zinsser-Hopkins strains as compared with 8.0 and 7.0 per cent for the Chinese III and IV strains respectively.

Eye lesions were not observed in the first New York experiment. In the sec-

ond, however, their proportionate values were 3.8 and 1.5 per cent for the Chinese IV and Nichols strains respectively (Table IV). The results of individual experiments, furthermore, showed generally higher proportionate values for the Chinese strains, the mean values for the American strains ranging from 1.5 to 5.6 per cent as compared with 3.8 to 11.1 per cent for the Chinese strains (Table IV).

Second, the infections produced by the Chinese strains and particularly the IV strain, showed a tendency toward slightly shorter incubation periods for the later phases of reaction (Table III). The mean incubation period of the metastatic orchitis was 46.7 days after inoculation for the Chinese IV strain, 6 days earlier than that of the Nichols and Chinese III strains and 10 days earlier than that of the Zinsser-Hopkins strain. The mean time of appearance of the first generalized lesion was 53.3 and 52.0 days after inoculation for the Chinese III and IV strains as compared with 57.2 and 60.9 days for the Nichols and Zinsser-Hopkins strains respectively. Similar differences occurred with respect to the mean time of appearance of the last generalized lesion and of all lesions. The difference for the duration of generalized disease activity is somewhat less marked as far as total mean values are concerned, but considering only the Peiping experiments, the mean values were 24.3 and 24.0 days for the Nichols and Zinsser-Hopkins strains and 18.6 and 20.5 days for the Chinese III and IV strains respectively.

Third, the tendency toward regression and healing of lesions was much greater with both Chinese strains. In the longer Peiping experiments the proportion of rabbits which were clinically negative at the conclusion of the experiments was 85.4 and 64.6 per cent for the Chinese III and IV strains as compared with 10.0 and 21.2 per cent for the Nichols and Zinsser-Hopkins strains respectively (Table V). The findings of the shorter and complicated New York experiments showed a similar tendency. It is probable that this acceleration of the latent phase was related to the shorter time periods of the later phases of the disease discussed above.

The results of the supplementary experiment in which the intradermal route of inoculation was used were in general agreement with those observed in the major part of the study in which the intratesticular route of inoculation was employed. The mean proportionate value of eye lesions was 3.3 and 11.7 per cent for the Chinese

III and IV strains as compared with 0.0 and 3.0 per cent for the Nichols and the Zinsser-Hopkins strains (Table VII). The mean time values for generalized disease activity tended to be shorter for the Chinese strains and the proportion of negative or healed rabbits at the end of the experiment was much higher (Table VII). In addition there was a general tendency for the primary chancres produced by the Chinese strains to be somewhat smaller, less destructive and less enduring than those produced by the American strains.

The predominant type of generalized manifestation with the Nichols strain (intratesticular inoculation) was involvement of the skin and mucous membranes. In three experiments slightly more than half and in the last experiment more than three-fourths the lesions were of this type (Table IV). In the two experiments with the Zinsser-Hopkins strain skin lesions represented slightly less than half the generalized manifestations and bone lesions were proportionately increased. The results for the Chinese strains were somewhat more variable but in general the Chinese III strain appeared to resemble the Zinsser-Hopkins strain with a tendency toward a higher proportion of bone than skin lesions. In two of the Chinese IV experiments bone and skin lesions were equally represented while in the third skin lesions greatly outnumbered those of the bones. But as has been said, eye lesions were a more frequent manifestation with both Chinese than with the American strains. This finding recalls the early experimental work of Nichols (6) and of Reasoner (7) in which involvement of the eyes was prone to occur with certain strains.

The predominance of one class of lesions over another seems to be primarily concerned with the immune reaction developed by the host. In an infection of average severity, the occurrence of well marked bone manifestations is apt to be associated with relatively few cutaneous lesions, while the development of numerous skin lesions is usually found in those animals with minor bone involvement. With the same strain of *pallidum*, considerable variation in the proportions of bone and skin lesions may be observed in individual rabbits of the same experiment and also in groups of rabbits. The liability of the eye to involvement is greater than that of most tissues since it is not protected to an equal extent by the general reaction that takes place in other parts of the body (8). It has been observed that these

lesions usually occur in advanced infections and are not commonly seen in animals that show a prompt and vigorous reaction except in cases in which the disease proves to be unusually severe. Both mild and severe types of reaction were represented in these experiments and no division of them on the basis of strains could be made. On the whole, the general level of disease severity in all the experiments was high, and it was remarkably constant for all four strains as indicated by the mean focal distribution rates for generalized lesions (Table II). In the present instance of the association of a comparatively high incidence of eye involvement with strains of *pallidum* of oriental origin, it is of interest to recall that lesions of the eyes in experimental syphilis bear some analogy to neurosyphilis of man on account of the circumstances surrounding their development.

A great deal of evidence has now been accumulated to show that various types of disease may be obtained by modifying the conditions under which the syphilitic infection is initiated. For example, such factors as the viability of the organisms, the route of inoculation, the sex and breed of rabbits employed, have a definite influence on the character of the disease. It is also affected by the season of the year, being always comparatively mild during the summer months while the periods of greatest severity are spring and fall. The pathogenic properties of a strain can also be modified to an extreme degree by simply varying the conditions of passage. Thus, the reaction of consecutive groups of rabbits inoculated intratesticularly with the Nichols strain by means of lymph node material obtained from rabbits similarly inoculated but which had long been clinically negative (late latency) was characterized by insignificant primary lesions and an almost total absence of generalized lesions (9).

It is clear that the disease picture is not fixed and predictable, but is rather a variable quantity, and that many conditions may favor or prevent the occurrence of lesions of any given class. Little is known of such conditions, of the nature of their operation or of the extent to which they may function, but the fact that they apparently act most frequently through the medium of the host does not rule out the possibility that the invading organism may also be affected.

The present experiments were designed to control as many variables as possible and thus to take into account the effect of possible modify-

ing factors. Large numbers of rabbits of various stocks were used and four experiments were carried out in two widely separated laboratories over a 2 year period. *The results show that in most respects the infections produced by two Chinese strains of T. pallidum were entirely comparable to those produced by the highly pathogenic Nichols and Zinsser-Hopkins strains of American origin. Nevertheless, a higher incidence of eye lesions and an acceleration of certain time relations of the reaction, notably the production of a higher proportion of latent* cases within the time limits of the experimental period, were observed with the Chinese strains.

It is obvious that the reactions to the Chinese and American strains were not wholly identical. To attribute to the Chinese strains a selective affinity for the eye or a particular capacity to induce early latency is to ignore what is known of the biology of syphilitic infections with its marked tendency toward variation. It is unlikely that the strains in question possess special pathogenic properties of an inherent and fixed nature by virtue of which the variable effects were induced. It is much more likely that these results were referable to some peculiarity in the reaction of the animals which these organisms aroused. That the same variation of clinical picture would be encountered again under quite different circumstances or that the same strains would fail to reproduce this variation is entirely possible.

SUMMARY AND CONCLUSIONS

Two oriental strains of *T. pallidum* of Chinese origin were compared with two well known occidental strains, the Nichols and the Zinsser-Hopkins, on the basis of the clinical reaction to infection in the rabbit. Four experiments were carried out, two each in Peiping and New York. Animals from American and Chinese sources were represented in each experiment.

The results obtained showed that the reaction to both Chinese strains was generally comparable to that of the American strains. But with both Chinese strains, lesions of the eyes were more frequent, the time relations of certain phases of the reaction were shortened, and there was a higher incidence of complete recovery (latency) within the observation period. In view of the well known variability of the syphilitic reaction, however, it was not felt that these qualitative

differences in clinical response could be ascribed to inherent or biologically fixed properties of the strains.

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JAPANESE B ENCEPHALITIS VIRUS: ITS DIFFERENTIATION FROM ST. LOUIS ENCEPHALITIS VIRUS AND RELATIONSHIP TO LOUPING ILL VIRUS

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PLATES 26 AND 27

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When encephalitis broke out in August and September of 1932 and 1933, centering in Illinois and Missouri, it was said to resemble Japanese B summer encephalitis (1). But after the St. Louis virus agent was discovered and found to be neutralized specifically by sera from convalescents, additional tests showed that this virus was not neutralized by sera from convalescents of Japanese B encephalitis (2). Further comparisons could not be made at that time because the etiological agent of Japanese B encephalitis had not yet been isolated, but the two diseases appeared to be immunologically distinct (2).

Japanese encephalitis recurred in epidemic form in 1934 and 1935, and from a number of cases virus was recovered. Hayashi, in 1934, reported the transmission of a virus from brain tissue of a fatal case to monkeys for several generations (3), and in 1935, October to December, Kawamura, Hashimoto, Kasahara, Kaneko, Takaki, Taniguchi, and Mitamura reported successful inoculations of brain tissue from fatal cases into mice, and occasionally into monkeys (4). Supplementing these early statements, further reports became available, in which the virus was related directly to the human disease (5–8).

The Japanese workers regarded their virus as similar in many respects to the St. Louis virus. Hence an exchange of Japanese and St. Louis strains and sera was effected between Drs. Kodama, Hashimoto, Takaki, Kasahara, and Mitamura and ourselves in the spring of 1936, and comparative studies were continued in Japan and in our laboratories.

More recently available reports from Japan (9–13) agreed that the

strains of Japanese virus recovered by various workers are identical; that they were neutralized by sera of a large percentage of tested convalescents and by sera of a few individuals without a history of encephalitis living in the epidemic areas, but not by sera of persons without encephalitis living in regions free of the disease (10, 11). Finally, they regarded the Japanese virus as generally similar but not identical with the St. Louis virus (9–11, 13).

The onset of the experimental disease in mice was said to be more frequently accompanied by paralysis of the posterior extremities (8, 9, 5, 7), and the virus following nasal instillation had a greater tendency to enter the blood stream (9) and a greater virulence when injected intraperitoneally (8, 9, 5, 7). In monkeys the virus was more virulent in that it induced a rapid and fatal cerebellar syndrome (9).

Kawamura and associates (9), and Kasahara and associates (13) found no cross-protection in hyperimmune Japanese and St. Louis rabbit sera nor in sera of convalescents of the two diseases. Kudo (10) and Takaki (11) observed no cross-protection of St. Louis hyperimmune rabbit sera but some crossing of Japanese immune rabbit sera.

The present report of our own studies on Japanese encephalitis, besides confirming for the most part those of the Japanese workers, shows certain relationships between this and other viruses associated with epidemic encephalitis in man.

Characteristic Reactions of Japanese Virus in Animal Species

The Japanese virus[1] induced reactions in animal species which were readily distinguished from those produced by St. Louis virus but approximated closely those of louping ill virus. It proved innocuous in rabbits and guinea pigs but induced in mice, monkeys, and sheep a fatal encephalitis.

Thus Swiss mice developed encephalitis following injections of virus by the intracerebral, intranasal, subcutaneous, and intraperitoneal routes. Following nasal instillation they showed, after 5 to 8 days, paralyses of the posterior extremities, or occasionally tremors and convulsions. They became prostrate and died in 7 to 10 days. The experimental disease resembled that following inoculation

[1] Six strains received from five Japanese investigators proved similar in all respects as tested in our laboratory. They passed Seitz filters readily, were virulent when inoculated in 0.03 cc. amounts intracerebrally into Swiss mice to the 10^{-7} dilution, and when frozen and dried retained their virulence well.

with louping ill virus and with St. Louis virus except for a more frequent onset with paralysis. Histological examination of tissue of these mice showed a type and distribution of lesions similar to those produced by St. Louis and louping ill viruses. The brain showed perivascular and subdural accumulations of round cells plus specific necrosis of nerve cells in the olfactory tracts, Ammon's horn (Fig. 1, and Kawamura, Fig. 4 (9)), anterior limbic area, hypothalamus, and, at late stages, throughout the cortex. The spinal cord remained relatively normal. Virus injected intraperitoneally or even subcutaneously in relatively small doses, 100 to 1,000 times the minimum intracerebral dose, usually induced encephalitis. Louping ill virus was similar in this respect but St. Louis virus was innocuous by these routes unless massive doses were employed. Moreover, Japanese and louping ill viruses, inoculated subcutaneously or intraperitoneally, reached the circulating blood promptly and persisted longer than St. Louis virus. Finally, Japanese and louping ill (14) viruses, following nasal instillation, were readily recovered from the blood stream, while St. Louis virus was rarely found.

Macacus rhesus monkeys were susceptible to the Japanese virus inoculated intracerebrally (8, 9, 5, 7) or intranasally. Following nasal instillation they showed an elevation of temperature to 106° on the 4th day, and a severe cerebellar ataxia on the 5th or 6th day. They became prostrate and died within 10 days. Samples of blood drawn daily and injected intracerebrally into mice failed to show virus. Sections taken at autopsy showed lesions limited primarily to the brain and consisting of necrotic nerve cells scattered irregularly, plus foci of round cells surrounding small blood vessels. Necrosis of the Purkinje cells of the cerebellum was especially marked (Fig. 2, and Kawamura, Fig. 14 (9)). These cells in the superficial convolutions appeared enlarged, or with pycnotic nuclei and granular cytoplasm, or shrunken with deep staining cytoplasm. In the deeper crypts many of the Purkinje cells were entirely missing and the few remaining were small and distorted. Nearby, a local "gliosis" was not uncommon. There was very little change in the surrounding tissue. The reactions described above following nasal instillation are generally similar to those following intracerebral injection of the virus. They are also similar to those following intracerebral inoculation of the louping ill virus (15). Monkeys injected with St. Louis virus, on the other hand, either remained normal or developed a mild, non-fatal encephalitis. Lesions could not be demonstrated with certainty without multiple intracerebral injections.

Sheep inoculated intracerebrally or intranasally with Japanese virus developed an acute, fatal encephalitis.

Lambs weighing 40 to 60 pounds received an intranasal instillation of 1 cc. of the mouse brain virus diluted 1 to 100. Temperatures rose on the 4th day to 106.0°, the animals became quiet, lost appetite, their heads drooped and legs weakened, and by the 8th day they were unable to rise. No definite central nervous system signs were noted. Blood drawn daily and injected intracerebrally into mice showed no virus. At autopsy, brain tissue injected into mice brought them down promptly with the characteristic signs of the Japanese disease. Sections of brain tissue showed blood vessels generally engorged and surrounded by

many round cells. Foci of round cells were likewise scattered irregularly throughout the brain. Many Purkinje cells of the cerebellum were in various stages of necrosis and neighboring glial cells appeared abnormal (Fig. 3). Scattered nerve cells throughout the cortex and brain stem were likewise necrotic. As with monkeys, the reactions described above following nasal instillation are generally similar to those following intracerebral injection of the virus. They are also similar to those following intranasal and intracerebral inoculation of the louping ill virus (Fig. 4).

1 cc. of virus diluted 1 to 10 and injected subcutaneously into lambs proved harmless but immunized them against a later nasal injection fatal to unvaccinated controls.

TABLE I

Virulence of Central Nervous System Viruses of Man Inoculated Intracerebrally into Laboratory Animals

Virus	Rabbit, Guinea pig	Mouse	Macacus monkey	Young sheep
Rabies	++	++	++	++
Louping ill; Japanese B encephalitis; Australian X (?)	0	++	++	++
St. Louis encephalitis	0	++	0–±	0
Poliomyelitis	0	0	+	0

These reactions in animal species differentiate the Japanese B from the St. Louis virus but relate it closely to louping ill virus.

A presumptive differential diagnosis of Japanese B encephalitis and other viruses associated with a primary encephalitis of man may be carried out on the basis of the above reactions in animal species in the manner outlined in Table I.

Lack of Cross-Resistance of Immunized Mice

Mice immune to Japanese virus were not immune to St. Louis virus, and conversely, mice immune to St. Louis virus were not immune to Japanese virus. Tests were not made with louping ill virus.

Mice were immunized against Japanese and St. Louis virus by repeated subcutaneous injections of sublethal doses of the virulent homologous virus. Attempts to immunize mice against louping ill virus failed. Sublethal doses, even when repeated, did not immunize while larger doses, lethal to 20 to 30 per cent of vaccinated mice, left a selected group of survivors whose subsequent resistance

to homologous or heterologous virus could not be judged as due to specific immunity factors alone, but might have depended on initial, non-specific, inherited factors. For this reason these survivors were not considered proper material for testing and were discarded.

The Japanese and St. Louis virus mice were tested after 2 to 4 weeks with homologous and heterologous virus in graded intracerebral doses. Unvaccinated mice were likewise tested as controls. The protocol of one such experiment is shown in Table II, in which the Japanese virus proved fatal to unvaccinated and to St. Louis vaccinated mice through the 10^{-6} dilution but not to mice immunized with Japanese beyond the 10^{-5} dilution. Similarly, the St. Louis virus was fatal alike to unvaccinated and Japanese virus mice through the 10^{-6} dilution but not to St. Louis virus mice beyond the 10^{-4} dilution.

TABLE II

Absence of Cross-Resistance of Mice to Japanese B and St. Louis Encephalitis Viruses

Mice vaccinated against	Vaccinated mice tested with	Duration of life of tested mice in days: 0.03 cc. of test virus diluted 10^{-5}	10^{-6}	10^{-7}
Unvaccinated controls	St. Louis virus	*5, 5, 5, 5	5, 5, 5, 6	†S, S, S, S
St. Louis virus	" " "	S, S, S, S	S, S, S, S	—
Japanese B virus	" " "	6, 6, 6, 7	5, 6, 6, 7	—
Unvaccinated controls	Japanese B virus	5, 5, 5	6, 6, 6, 7	7, 7, 8, S
Japanese B virus	" " "	8, 8, 8, 9	S, S, S, S	—
St. Louis virus	" " "	5, 5, 5, 5	7, 8, 9, S	—

* Mouse died of encephalitis 5 days following injection.

† Mouse remained well following injection. Discarded at 30 days.

Lack of Cross-Protection of Immune Sera

Protection tests disclosed no definite immune relation between Japanese B, St. Louis, and louping ill viruses.

Tests with hyperimmune sera were made in the following manner.

Monkeys were each given repeated subcutaneous and intraperitoneal injections of the respective living mouse brain viruses. Sera were drawn and mixed undiluted with various concentrations of mouse brain virus prepared in the usual manner in broth as a diluent. After standing at 37°C. for 2 hours and at 23°C. for 2 hours, the mixtures were each injected intracerebrally in 0.03 cc. amounts into four Swiss mice. Duration of life of the injected animals was recorded in days.

TABLE III

Lack of Cross-Neutralization of Japanese and St. Louis Encephalitis, and Louping Ill Viruses in Heterologous Sera

Test sera		Test virus	Duration of life of tested mice in days. Dilution of virus in serum mixtures. 0.03 cc. injected			
			10^{-4}	10^{-5}	10^{-6}	10^{-7}
Monkey	Normal	Japanese No. 2	*5, 5, 5, 7	7, 7, 8, 8	8, 8, 9	7, 7, 12
"	Japanese No. 2 immune	" " "	9, 11	S, S, S, S	S, S, S	—
"	St. Louis immune	" " "	—	7, 7, 8, 10	7, 8, 10	—
"	Louping ill immune	" " "	—	8, 9, 10, S	10, 11, S	—
"	Normal	St. Louis	—	5, 5, 7, 7	10, 10, 10, 11	
"	St. Louis immune	" "	7, 7, 9	S, S, S, S	—	—
"	Japanese No. 2 immune	" "	—	9, 9, 10, 10	S, S, S, S	
"	Louping ill immune	" "	—	7, 9, 9, 9	11, 11, S, S	
"	Normal	Louping ill	7, 7, 7, 7	7, 8, 8, 8	9, 9, 9, 10	8, 9, 9
"	Louping ill immune	" "	S, S, S, S	S, S, S, S	S, S, S, S	—
"	Japanese No. 2 immune	" "	—	7, 8, 9, 9	9, 9, 11, 11	
"	St. Louis immune	" "	—	9, 9, 9, 9	8, 9, 9, 9	

S = mouse remained well 21 days.

— = dilution not tested.

* Mouse died of encephalitis 5 days following injection.

The protocol summarized in Table III shows Japanese virus No. 2 plus normal monkey serum fatal to 50 per cent or more of four mice per dilution through 10^{-7}, fatal through 10^{-4} when combined with homologous immune serum, and through 10^{-6} when mixed with St. Louis and louping ill immune sera respectively. Similarly, St. Louis virus mixed with normal monkey serum was fatal through the 10^{-6} dilution, with homologous immune serum through the 10^{-4} dilution, and with Japanese or louping ill, through the 10^{-5} and 10^{-6} dilutions respectively. Finally, louping ill virus mixed with normal monkey serum was fatal through the 10^{-7} dilution, with homologous immune serum, in less than the 10^{-4} dilution, and with Japanese and St. Louis sera through the 10^{-6} dilutions, respectively. In short, virus mixed with heterologous immune sera, although slightly less active than with normal monkey serum, was 10 to 100 times more active than when combined with homologous immune sera.

Sera from St. Louis convalescents did not protect against the Japanese virus nor did sera from Japanese convalescents protect against St. Louis virus. A curious instance of cross-protection, however, has been noted by Kuttner and confirmed in our laboratory.

Kuttner described two cases of encephalitis contracted by Europeans in China (16). Serum from W. protected against both Japanese and St. Louis viruses, and serum from G. protected against Japanese but was not tested against St. Louis virus. Further sera from these cases[2] were sent directly to our laboratory and were found to protect well against both Japanese and St. Louis and not against louping ill virus.

Finally, sera from Japanese and St. Louis convalescents did not protect against louping ill virus nor did sera from supposed convalescents of louping ill virus (17) protect against Japanese or St. Louis viruses.

DISCUSSION

The virus from Japan described above is regarded as the etiological agent of Japanese B summer encephalitis on the following grounds. During an epidemic outbreak, strains were recovered from brain tissue of fatal cases of encephalitis by several independent investigators in Japan at the same time and by the same technique. All strains have proved identical in so far as tested. They were said to be neutralized specifically by sera from convalescents and certain contacts. The virus proved similar to others in the encephalitis group

[2] Obtained through the kindness of Dr. Kuttner.

and yet ultimately distinct. Japanese B encephalitis is included, therefore, in the group of primary central nervous system infections of man of known virus etiology which occur in epidemic form in late summer. These infections, poliomyelitis, Japanese B encephalitis, Australian X disease, and St. Louis encephalitis, have features in common but are distinguishable by laboratory tests. Outbreaks of each are frequent and limited chiefly to hot weather, and cases of each are scattered throughout an infected community, usually not more than one per family. Clinically the diseases are often difficult to identify. Each may be recognized, however, by testing sera of convalescents for specific neutralizing properties against one of these virus agents, or by obtaining virus from brain tissue of fatal cases and testing its virulence for mice, monkeys, and sheep and its neutralization in specific antisera.

Possible relationships between Japanese B encephalitis, the sheep encephalitis of Scotland (louping ill), and polioencephalitis of children in Australia (X disease) should be further explored. The sheep virus is probably infectious for man, judging from the fact that three investigators, shortly after commencing work with the virus (17), contracted encephalitis and later showed specific neutralizing antibodies in their sera. Moreover, louping ill virus has been related to the virus associated with X disease of children in Australia (18), and now has proved similar to the newly discovered Japanese B encephalitis virus. Further studies are needed to determine whether this relationship is merely superficial or of immediate epidemiological importance.

The question of mode of spread of this group of infections through a community and their portal of entry into the body are unknown and difficult to investigate. Laboratory tests on susceptible animals indicate clearly that the most vulnerable portal for the experimental introduction of these viruses is the nasal mucosa: this is the route of choice in mice for the St. Louis virus; in mice, monkeys, and sheep for the Japanese and louping ill (21) viruses; and in monkeys for the poliomyelitis virus. There is also evidence that spontaneous louping ill may occur both in man (17) and in mice (20) through the nasal mucosa. But contact experiments to induce spontaneous transfer of St. Louis, Japanese B, and poliomyelitis infections among susceptible

laboratory animals have failed and it is difficult or impossible to detect the virus in the upper respiratory tract of the diseased individual. On the other hand, subcutaneous injections of Japanese B and louping ill viruses in mice and of poliomyelitis virus in monkeys and possibly man may also induce disease. Moreover, reports state that in nature louping ill infection of sheep takes place subcutaneously by the bite of an insect (19). But experiments to detect virus in the blood of individuals following natural or experimental infection are either positive for brief periods only (louping ill, Japanese B viruses) or are completely negative (poliomyelitis, St. Louis viruses). Hence there is evidence for and against both the upper respiratory and subcutaneous routes of transmission of these infections and the question remains perplexing.

CONCLUSIONS

1. Japanese B encephalitis virus, obtained from Japanese investigators, has proved virulent for mice and monkeys, confirming the reports from Japan. It has also been found virulent for monkeys when instilled intranasally and for sheep when introduced intracerebrally or intranasally.
2. Japanese B encephalitis virus has been differentiated from St. Louis virus and found similar to louping ill virus according to its reactions in animal species. Serologically, however, it is distinct.
3. Japanese B encephalitis and its related group of primary virus encephalitides of man have been discussed with regard to their differentiation and mode of spread.

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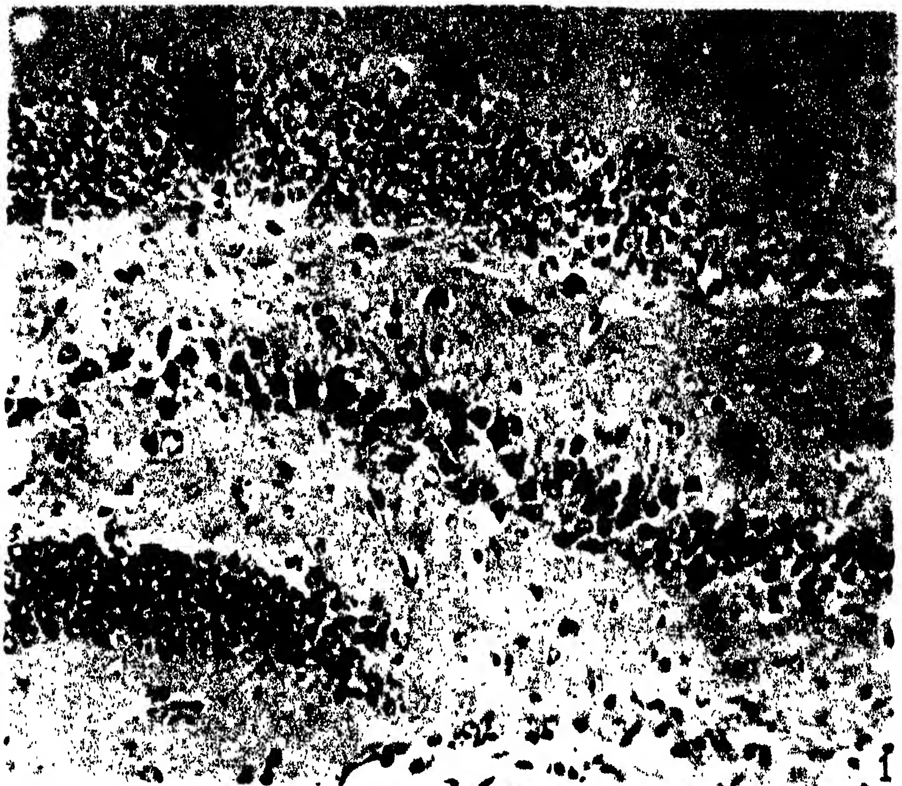
EXPLANATION OF PLATES

Plate 26

Fig. 1. Swiss mouse. Section through Ammon's horn 6 days after nasal instillation of Japanese B encephalitis virus. The left and right vertical columns of pyramidal cells appear relatively normal. Cells in the central column are in various stages of necrosis. Eosin-methylene blue. × 275.

Fig. 2. Macacus monkey. Section through cerebellum 8 days after nasal instillation of Japanese B encephalitis virus. The Purkinje cells are necrotic or entirely absent. Eosin-methylene blue. × 275.



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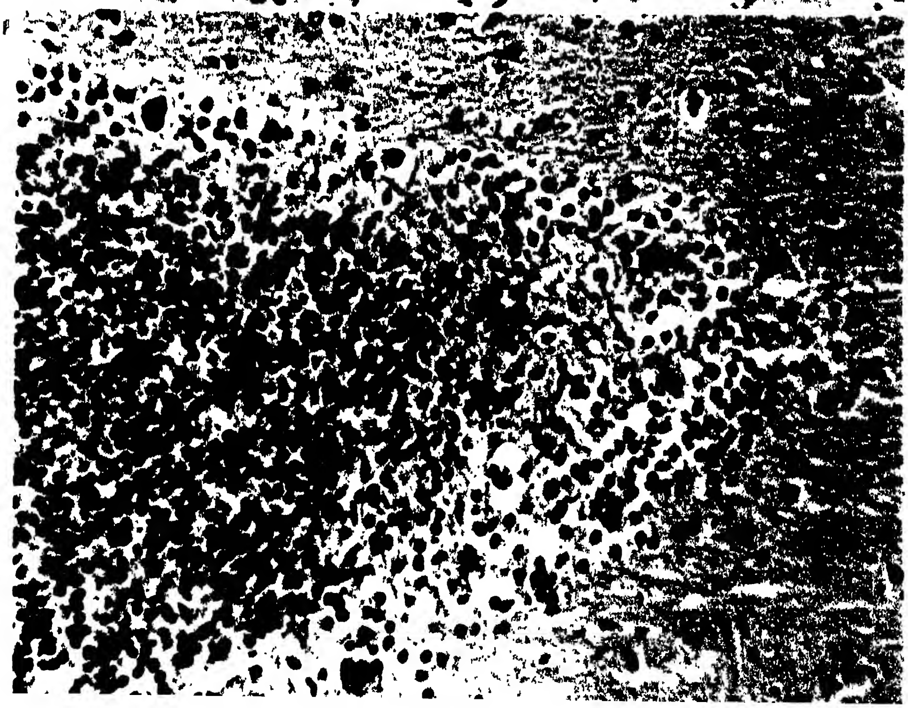
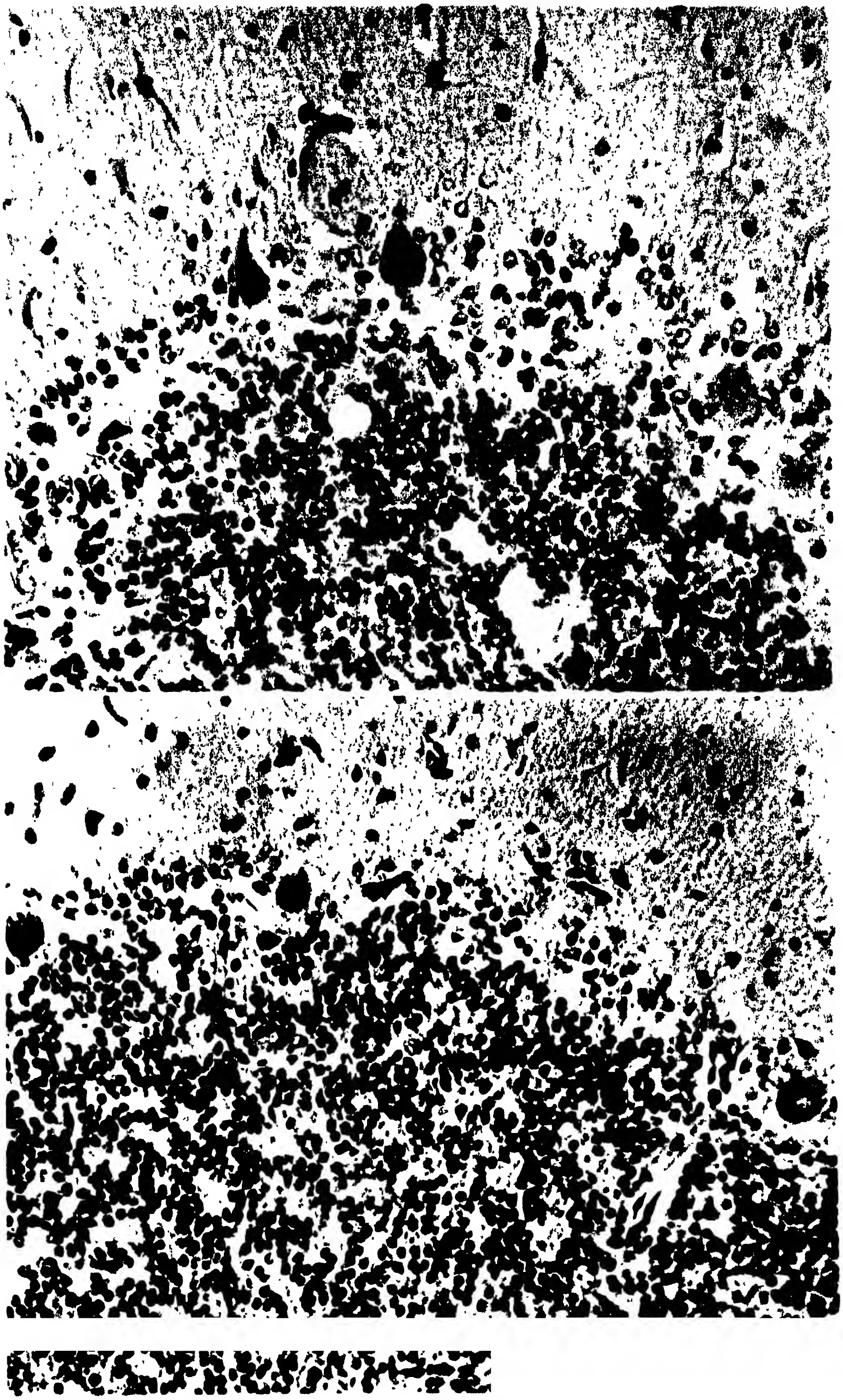


Plate 27

Fig. 3. Young sheep. Section through cerebellum 9 days after nasal instillation of Japanese B encephalitis virus. The Purkinje cells are in early stages of necrosis. A few are missing entirely. There is some reaction of glial cells in the molecular layer. Eosin-methylene blue. × 275.

Fig. 4. Young sheep. Section through cerebellum 8 days after nasal instillation of louping ill virus. The Purkinje cells are necrotic and there is reaction of neighboring glial cells. Eosin-methylene blue. × 275.



Photographed by Joseph B. Haulenbeek

(Webster: Japanese B encephalitis virus)

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LIBERATION OF ACETYLCHOLINE BY THE SUPERIOR CERVICAL SYMPATHETIC GANGLION AND THE NODOSUM GANGLION OF THE VAGUS[1]

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The formation of a chemical substance at the terminals of the primary neurons is implied in the theories of humoral transmission, although these structures do not have the properties ordinarily associated with secreting organs. In order to surmount the difficulty which is here presented, Lapicque has proposed for muscle that the acetylcholine (A. Ch.) may be derived from the nucleated sole plate, rather than from the endings. An analogous source for the substance in sympathetic ganglion would be the satellite cells, with which the nerve terminals and the dendrites of the ganglion cells seem to be in intimate contact (de Castro, 1926, figs. 3, 6, 13, 16, 19). Experiments have been performed to test this possibility.

As satellite cells are in contact with sensory cells as well as with ganglion cells, obviously the first experiment to be tried in order to test the hypothesis was to look for the production of A. Ch. after stimulation of the vagus ganglion or antidromic stimulation of a sympathetic ganglion. When the experiment was performed, the substance was found; but the conditions of its appearance were such as to suggest it to be a product of metabolism, rather than an agent produced exclusively in a specialized structure for a specific purpose.

The superior cervical sympathetic ganglion and nodosum ganglion of the vagus were perfused in the manner described by Kibjakow (1932), Feldberg and Gaddum (1934), Feldberg and Vartiainen (1934), von Brücke (1935), and Brown and Feldberg (1936 a, b); and the output of A. Ch. was determined with the leech preparation described by Fühner (1918), Minz (1932), and Chang and Gaddum (1933).

Four series of experiments were made, with changes in the technique

[1] The experiments here reported were planned with Dr. H. S. Gasser, who had suggested as a working hypothesis the possibility of the liberation of A.Ch. by satellite cells. Doctor Gasser has taken part in a number of the experiments and offered helpful criticisms of others, for which the author is deeply indebted. Unfortunately, Doctor Gasser has been prevented from following the research in detail, and the present author must take full responsibility for the statements made in this paper.

in each series. All experiments were performed on cats anesthetized with Dial (0.6-0.7 cc. per kgm.).

First series of experiments. *Technique.* The experiments of the first series followed in every detail the method described by Feldberg and Gaddum (1934) and Feldberg and Vartiainen (1934). A minor change only was made. In order to obtain a larger operating field, the larynx and base of the tongue were extirpated after ligature of the lingual and superior laryngeal arteries on both sides. Supramaximal rhythmic condenser discharges at 4 to 5 per second were employed for stimulation, and their efficacy was checked by observation of the movements of the pupil and nictitating membrane. The bath for the leech preparation was made as small as possible, only 0.7 to 0.8 cc. of fluid being necessary to fill it when the glass holder and the strip (about 15 to 20 segments) of muscle were in position. During the periods between tests, oxygenated plain or eserinized Locke's solution was kept running through the bath.

Results. The first series included seven successful perfusions (I-VII). Three of the experiments (II, IV and VII) yielded results as described by Feldberg and Vartiainen. The specimens of perfusate collected during periods of postganglionic stimulation or of stimulation of the vagus were either inactive, or had the same activity on the leech muscle as control samples collected during periods when there was no stimulation. But the specimens obtained during preganglionic stimulation were always extremely active, two of them producing an effect greater than the A. Ch. solutions at 250 and 200γ per liter respectively. Furthermore it was observed, in agreement with Feldberg and Vartiainen (1934, p. 109), that preganglionic stimulation, delivered after a period of postganglionic tetanus, failed to produce a peripheral response, although large amounts of A. Ch. were released.

In the other four experiments different results were obtained, as considerable amounts of A. Ch. were liberated by postganglionic stimulation. Experiment V (2/9/1937) is typical of this group. The specimens were tested on the leech at various dilutions, 1:1.4 for samples 1, 2, and 4; 1:2.8 for samples 5, 6, and 9; and 1:4.2 for samples 7 and 10. Specimens 3 and 8 were not tested. The sensitivity of the leech strips was assayed with A. Ch. solutions at 5, 10, and 20γ per liter.

Examination of the records shows that at the start of the perfusion (fig. 1, *1 c*, and *3 c*) there was a spontaneous output of A. Ch. which lasted until control sample 4 was collected. The first period of postganglionic stimulation (5) caused liberation of A. Ch. in very nearly the same concentration as was produced by preganglionic stimulation in period 10 (about 80 to 100γ per liter); and after the perfusate had again become negative (sample 6), a second period of postganglionic stimulation (fig. 1, 7 A) likewise brought out a considerable amount of the substance.

Following these procedures it was found that preganglionic stimulation no longer evoked responses of the pupil and nictitating membrane, although A. Ch. was produced in the ganglion (period 10) and the previous postganglionic stimulation had produced maximal contractions.

Inasmuch as the appearance of A. Ch. following postganglionic stimulation was contrary to the observations of Feldberg and Vartiainen, the possibility of direct spread of the stimulating current from the postganglionic to the preganglionic fibers had to be considered. But this explanation was proven to be untenable when the action potentials in the preganglionic fibers were observed oscillographically. Even with the employment of shocks much stronger than were used routinely, no escape occurred. Consequently, the liberation of A. Ch. by impulses conducted antidromically to the ganglion cells had to be regarded as a genuine phenomenon

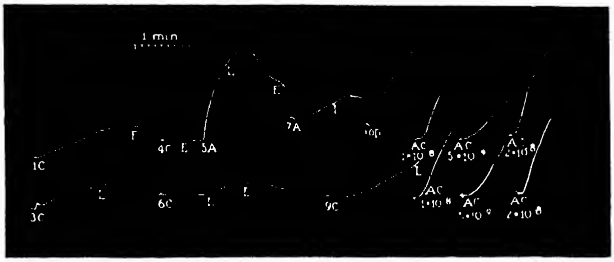

Fig. 1. Responses of the leech to the perfusate and to standard solutions of A.Ch. (10.5 and 20γ per liter). Experiment V (2/9/1937). The numbers on the record are those of the samples of perfusate. *C*, control; *A*, antidromic stimulation; *D*, preganglionic tetanus. Each horizontal series contains responses of the same strip of leech muscle. *L* and *E* indicate the changes of the bath to Locke's solution and eserinised Locke's solution. Dilutions of the samples indicated in text. In this and the following figures, 1 min. represents one of the small divisions of the time scale.

and it was thought, in agreement with an assumption of Eccles (1936, p. 363), that for some reason the antidromic impulses in the negative experiments had been blocked somewhere on their way to the ganglion. This possibility was examined in the next experiments.

Second series of experiments. *Technique.* The operation was made as previously described, except that at the end of the dissection the bulla ossea was opened and its medial wall extirpated. The IX, X, XI and XII nerves were cut, and the internal carotid artery was severed between ligatures. The blood vessels under the postganglionic trunk were also ligated and divided. The perfusion was made in the usual manner; but in three experiments (VIII, IX, and X), immediately after starting the perfusion, the cat was killed by bleeding. The temperature in and around the ganglion was carefully maintained at 37 to 38 degrees C.

Results. The most striking result was the appearance in the perfusate of large amounts of A. Ch. in the absence of stimulation. In one case (VIII, 3/3/1937), the activity of the fluid collected without stimulation at the end of the experiment, when the rate of perfusion had become very slow, was greater than that during the preceding preganglionic stimulations. In fact, the activity of these control samples was estimated at 250 to 300γ A. Ch. per liter. In all four experiments of this series (VIII–XI) postganglionic stimulation was ineffective in liberating A. Ch.

In experiment XI (3/2/1937) the cat was kept alive. Figure 2 reproduces the responses of the leech to the samples of perfusate diluted 1:2.8 and to a solution of A. Ch. at 100γ per liter. Only one-half of the leech

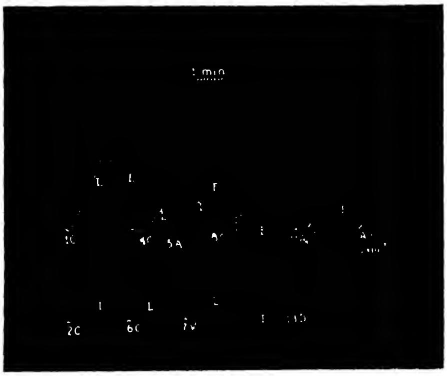

Fig. 2

Fig. 3

Fig. 2. Responses of the leech to the perfusate and to a standard solution of A.Ch. (100γ per liter). Experiment XI (3/2/1937). The kink in the curve 13*D* was due to a wrinkle in the smoked paper. *V*, stimulation of the vagus. Dilution of the samples indicated in text.

Fig. 3. Responses of the leech to the perfusate obtained during a control period (6*C*); and two periods of vagus stimulation (4*V* and 7*V*) and a standard solution of A.Ch. (10γ per liter). Dilution of the perfusate 1:1.4. Experiment XV, (4/2/1937).

was assayed; according to the results of the tests the other half was less sensitive.

During the early part of the perfusion, the ganglion, even when it was not stimulated, yielded considerable A. Ch. As little physiological activity was to be expected on account of the deep narcosis, and as discharge from cut nerve fibers was impossible,—since the preganglionic nerve had not yet been divided,—the outpouring must have been attributable to the condition of the ganglion itself. As the experiment proceeded the spontaneous production diminished, and it was not visibly augmented when the preganglionic trunk was cut.

Neither stimulation of the postganglionic trunk (5 *A*, 11 *A*) nor of the vagus nerve (7 *V*) affected the output of A. Ch.; but preganglionic stimu-

lation augmented it to a marked degree (13 *D*), the concentration in the perfusate being estimated at over 250γ per liter. In the course of the observations it was noted that, while postganglionic stimulation during periods 5 and 7 caused maximal motor responses, preganglionic stimulation during period 13 was entirely ineffective. In order to locate the position of the block, strongly supermaximal stimuli were applied directly to the ganglion. As no response was evoked, it became clear that during the perfusion a block had developed somewhere between the ganglion cells and the postganglionic fibers. Thus no impulses could pass from the preganglionic fibers to the periphery, nor could any pass antidromically into the ganglion to effect a release of A. Ch.

When the ganglion was examined with a binocular microscope at the end of the perfusion, the capillaries in the distal part of the ganglion were seen to be filled with blood; that is, they had not been perfused and possibly an asphyxial block had developed. Accordingly the operative technique was modified.

Third series of experiments. *Technique.* The principal innovation in the method of preparation was not to cut the vagus nerve central to the ganglia, and thereby avoid the production of a stump which could retract and shut off the small venous capillaries in the neighborhood of the ganglia. Instead, after the bulla ossea had been opened, a silk loop was passed from the middle ear under the vagus nerve and the jugular vein, and then again under the postganglionic trunk, in a way so that when tied it interrupted the circulation through the internal carotid artery and the vessels accompanying the bundle of nerves, without interfering with the capillaries to the ganglion. Serial specimens of the perfusate were collected in amounts of 1 to 1.5 cc. (5–10 min.); but only selected samples were tested. The amounts of A. Ch. obtained in this series were small, and accordingly the frequency of stimulation was increased to 10 shocks per second.

Results. In experiment XII (3/3/1937) nine specimens were collected and five were tested on the same strip of leech. Sample 6 was collected before starting stimulation. In dilution of 1:1.72 it caused a slight contraction of the leech; sample 7 (postganglionic stimulation) in dilution of 1:2.8 produced a marked contraction; control sample 8 in dilution of 1:2.8 was entirely inactive; sample 9, obtained during stimulation of the vagus, was tested in dilution of 1:2.8 and found to be active, considerably more active than 6 (at 1:1.72 dilution), but less active than 7 at the same dilution. Finally sample 10 was collected during a preganglionic tetanus which caused maximal opening of the pupil and retraction of the nictitating membrane. It was tested in 1:7 dilution and found to be highly active.

Two facts were brought to light by this experiment: 1, that a postganglionic tetanization does not prevent a later transmission of preganglionic impulses through the ganglion, provided that the perfusion be made with

a conservative technique; and 2, that A. Ch. may be liberated not only by the antidromic conduction of impulses into the ganglion cells, but also by the passage of impulses through the nodosum ganglion of the vagus.

In the other three experiments of this series (XIII, XIV, and XV) stimulation of the vagus nerve in the neck also caused liberation of considerable amounts of A. Ch., but not with regularity. Thus in experiment XIII (3/16/1937) the vagus was stimulated during periods 7 and 16 and in both cases a large output of A. Ch. was recorded, while the control samples obtained immediately before and after stimulation were either inactive or only slightly active. In experiment XIV (3/17/1937) the vagus was stimulated during periods 5, 8, and 12. Samples 8 and 12 proved to be inactive, but sample 5 had the activity of A. Ch. $1:1 \times 10^8$, while control samples 4 and 7 were practically inactive. Again in experiment XV (4/2/1937) vagus sample 4 had a slight activity (fig. 3, 4 *V*); control sample 6 (fig. 3, 6 *C*) was inactive; but vagus sample 7 (fig. 3, 7 *V*) in dilution of 1:1.4 had an activity almost matching A. Ch. $1:1 \times 10^8$.

In view of the irregularity, the technique was again improved, this time in order to insure normal blood circulation through the ganglia until the very start of the perfusion.

Fourth series of experiments. *Technique.* The operation was performed in a manner so that no large blood vessels had to be ligated until after the start of the perfusion. All the organs in the neck and mouth (glands, larynx, tongue) and the prevertebral and other muscles were successively extirpated after individual ligature of their blood vessels, often of capillary caliber (the operation was carried out under a binocular microscope). At the end nothing remained in the neck but the carotid arteries, the jugular vein, carotid glomus, vagosympathetic trunk, the two ganglia, and segments of the IX, XI, and XII nerves. After injection of a small amount of anticoagulant (Liquoid), a cannula was inserted into the common carotid artery and the perfusion started. A few seconds later, when the blood had been washed out, the main vessels were ligated and a collecting cannula was inserted into the jugulovertebral anastomotic vein. From four to five hours were needed for the operation, as over one hundred blood vessels had to be tied. In order to prevent cooling of the tissues, the room was kept at 34 degrees C. and the region of the field of operation was maintained at 37 to 38 degrees C., with the humidity at saturation by means of a vaporizer. Four of the experiments were so successful that histological sections of the perfused ganglia could not be distinguished from those from control ganglia which had not been perfused.

As in the previous group of experiments, the perfusate was collected in serial samples, and with but few exceptions all the samples were tested. The whole series was kept in the ice box at 0 degree C. until the end of the perfusion, as it had previously been shown that the storage would

not cause a detectable loss of activity (see fig. 9, 22 *C*). The series was then divided into two groups of consecutive samples, numbers 1 to 14, for example, making up one group and numbers 15 to 30 the other; and each group was tested on its own member of the paired leech muscle preparations. The slope of the contraction of the leech muscle was all that was necessary for a rough estimate of the A. Ch. content of the samples, and this could be determined after a few minutes of contact of the perfusate with the preparation. At the end of this period the leech was washed with running Locke's solution for several minutes. Then, after further washing with eserinized Locke's solution for 8 to 10 minutes, it was ready for the next test. Needless to say, contamination of the perfusate was prevented by using individual pipettes for each sample and by carefully avoiding contact of the fluid with the fingers of the experimenter (Dale and Feldberg, 1934).

Results. The fourth series included six experiments, five of which will be described and discussed individually. This is necessary because, despite all the precautions, the liberation of A. Ch. turned out not to be as regular a phenomenon as was anticipated from the descriptions of Feldberg and Gaddum, Feldberg and Vartiainen, and Brown and Feldberg. The liberation was even more irregular than had been suggested by the experiments of the three previous series.

Experiment XVI (4/7/1937). The control samples were collected at five minute intervals; but in stimulation periods the collection lasted for six minutes (five minutes of stimulation and one more minute without stimulation). All the samples but two (4 and 7, controls) were tested, all in 1:1.4 dilution. The sensitivity of the leech strips was assayed with A. Ch. at 5 and 10γ per liter.

Figure 4 reproduces the results of the tests. During the first 25 minutes of perfusion (samples 1 to 5) no A. Ch. was liberated, despite the section of the preganglionic trunk immediately before the start of the perfusion, and although there was one period of vagus stimulation (5). Evidently spontaneous liberation of A. Ch. can be reduced to a negligible amount or prevented altogether by the use of careful operative technique; and when it occurs, it cannot adequately be explained on the basis of mechanical injury of the preganglionic trunk.

Sample 6 obtained during stimulation of the vagus nerve began to contain traces of A. Ch., and a larger amount was obtained in the following sample, 8. Also a larger amount was obtained during the next stimulation of the vagus at 9 and in the following period 10. Sample 11, a control tested on the less sensitive half of the leech, was negative, as was also sample 12 from vagus stimulation; but the latter was succeeded by two samples, 13 and 14, which indicated a large amount of A. Ch. The next vagus stimulation at 15 came after the large output at 14, and it is difficult

to tell how much of the amount produced is in continuation of the latter and how much is new production. In this instance at least there was no delayed output, as the perfusate became negative at 17. At this point, preganglionic stimulation at 18 brought out large amounts of A. Ch. The production continued into 19, and after falling to a low point at 20, a markedly delayed production occurred during 21 and 22, more than 10 minutes after the end of the preganglionic tetanus.

According to the histological evidence, the experiment was a successful one. The ganglion and the unperfused control from the opposite side were stained according to Bodian and counterstained with acid fuchsin; and the two presented the same histological picture. Nevertheless there was a difference between the end of the experiment and the beginning,

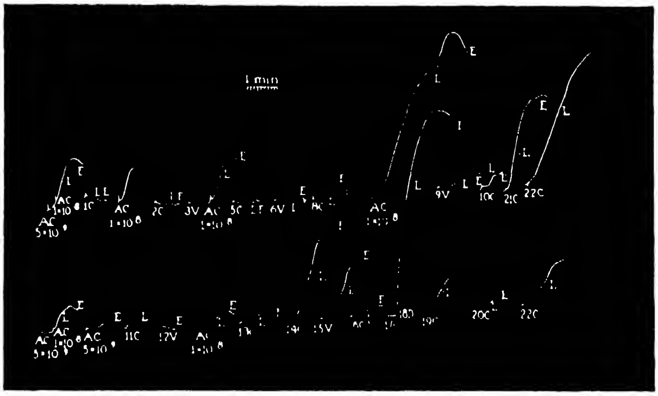

Fig. 4. Responses of the leech to the perfusate and to standard solutions of A.Ch. (5 and 10γ per liter). Experiment XVI (4/7/1937). Sample 22(C) was tested in both halves of the leech. Note that the solutions of A.Ch. are tested several times to detect changes in the sensitivity of the leech. Dilution of the perfusate 1:1.4.

for vagus stimulation evoked no A. Ch. at the beginning, not even a delayed production, while at the end a delayed liberation took place. Preganglionic stimulation differed from the vagus in that the output was larger and immediate as well as delayed.

Experiment XVII (4/8/1937). Twenty-four samples were collected and all were tested. Unless otherwise indicated, the collection periods were 5 minutes for the control samples and 6 minutes (5 minutes with stimulation and 1 minute without stimulation) for those with stimulation. The perfusion was carried out at a pressure of 60 cm. of water and the volumes of the samples were recorded. During the experiment the perfusion rate fell progressively to one-half of its original value. Although not as accurately measured, a falling off also occurred during all the other experiments, meaning no doubt that perfusion always occasioned some injury. No correlation between the appearance of A. Ch. and the rate

of perfusion could be established. All samples were tested in a 1:1.4 dilution, with leeches not very sensitive. The results of the tests are reproduced in figure 5 and compared with solutions of A. Ch. at 10, 50, and 100γ per liter. Table 1 summarizes the results of the experiment.

Only one preganglionic stimulation was tried (21). It brought forth A. Ch. promptly and in large amounts, the production outlasting the tetanus by 5 to 10 minutes. Antidromic tetanization during periods 2, 5, 8, and 14 caused only a delayed output of A. Ch. starting at least several minutes after the beginning of stimulation. Twice the production lasted only for a few minutes (samples 4 and 6), but in one case it lasted for 15 minutes (samples 15, 16, and 17). In one instance antidromic stimulation caused liberation of A. Ch. in two bursts, an initial one (sample 11) and a delayed one (sample 13). It was evident in every case that if the periods

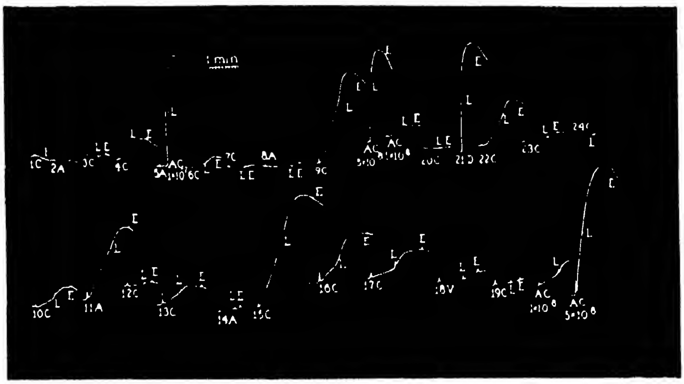

Fig. 5. Responses of the leech to the perfusate and to standard solutions of A.Ch. (10, 50 and 100γ per liter). Experiment XVII (4/8/1937). Dilution of the perfusate 1:1.4.

of collection of perfusate had been but slightly longer, the release of A. Ch. would have seemed to coincide in time with the stimulation.

Experiment XVIII (4/10/1937). In this experiment the effectiveness of preganglionic stimulation was checked by careful observation of the movements of the pupil and the nictitating membrane. Since the cat was under Dial narcosis, the pupil once opened never closed entirely, but the start of the opening and the start of the closing were easily detected. During every period of preganglionic stimulation the nictitating membrane was so retracted that it could scarcely be reached with a forceps, while during the control periods after it had been drawn out with a forceps it was found to be completely relaxed. The strength of stimulation necessary for threshold opening of the pupil was determined before starting the perfusion. During the experiment shocks from five to nine times this strength were used. From oscillographic studies it is known that even

the weakest of these strengths was maximal for the preganglionic trunk, and in fact the response of the pupil and the nictitating membrane were

TABLE 1

Experiment XVII (4/8/1937)

SAMPLE	DURATION OF COLLECTION	AMOUNT OF FLUID	APPROXIMATE CONCENTRATION OF ACETYLCHOLINE	SAMPLE	DURATION OF COLLECTION	AMOUNT OF FLUID	APPROXIMATE CONCENTRATION OF ACETYLCHOLINE
	minutes	*cc.*	*γ per liter*		*minutes*	*cc.*	*γ per liter*
1C	5	1.1	0	13C	5	0.8	10
2A	6	1.2	0	14A	6	0.9	2(?)
3C	5	0.9	0	15C	5	0.7	35
4C	5	0.9	20	16C	5	0.7	15
5A	6	1.1	0	17C	5	0.7	6
6C	5	0.8	10	18V	6	0.8	2(?)
7C	5	0.8		19C	5	0.7	0
8A	6	1.0	0	20C	6	0.8	0
9C	5	0.8	30	21D	6	0.8	100
10C	5	0.8	5	22C	7	0.9	20
11A	6	1.0	30	23C	7	0.7	5
12C	5	?	0	24C	10	1.1	0

TABLE 2

Experiment XVIII (4/10/1937)

SAMPLE	DURATION OF COLLECTION	AMOUNT OF FLUID	APPROXIMATE CONCENTRATION OF ACETYLCHOLINE	SAMPLE	DURATION OF COLLECTION	AMOUNT OF FLUID	APPROXIMATE CONCENTRATION OF ACETYLCHOLINE
	minutes	*cc.*	*γ per liter*		*minutes*	*cc.*	*γ per liter*
1C	4	1.5	2	19D	4	1.1	12
2D	2	0.7	10	20C	3	1.0	4
3C	3	1.1	1	21C	3	1.0	6
4C	3	0.9	2	22C	3	0.9	6
5C	3	1.0	0	23A	3	0.9	6
6D	2½	0.7	Less than 2	24C	3	0.8	6
7C	3	0.8	3	25C	3	0.8	3
8C	?	0.9	5	26D	2½	0.8	12
9C	3	0.8	3	27C	3	0.8	12
10D	3	0.7	10	28C	3	1.0	10
11C	4	1.2	2	29A	3	0.7	10
12C	4	1.2	Not tested	30C	?	1.0	12
13C	4	1.1	Not tested	31C	3	0.8	Less than 5
17C	3½	1.1	2	32D	4	0.8	10
18C	3	1.0	5	33C	3	0.7	5
				34C	4	0.8	Less than 5

always maximal. The frequency of stimulation was 10 shocks per second. The perfusion pressure was raised to 80 cm., so that a sufficient amount

of fluid could be collected in from 2 to 3 minutes. The amounts of fluid are indicated below. During period 14 (antidromic stimulation) escape of stimulus to the IX and X nerves caused reflex movements of the cat and displacement of the cannula with a resultant temporary interruption of the perfusion. The leech strips were of high sensitivity at the beginning of the tests, but the sensitivity as shown by frequent calibration with a standard solution of A. Ch. at 10γ per liter dropped progressively to about 50 per cent of its initial value. The results of the tests are reproduced in figure 6 and table 3, which contain a summary of the findings. The preganglionic tetanus lasted for one minute in periods 2, 6, 10, and 26, and for two minutes in periods 19 and 32. Antidromic stimulation lasted for three minutes in period 23 and for two minutes in period 29.

It is not necessary to emphasize that, because of the progressive loss in sensitivity of the leech, the concentrations of A. Ch. recorded in the

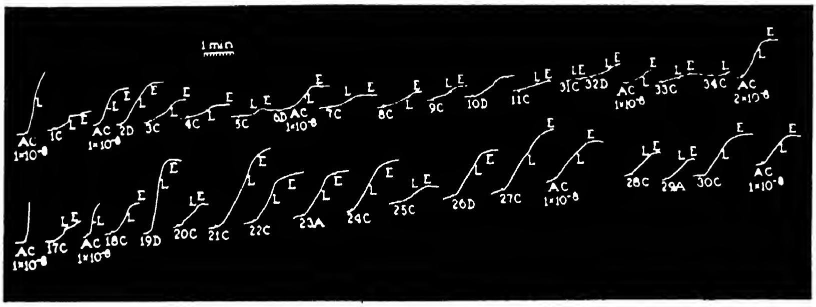

Fig. 6. Responses of the leech to the perfusate and to standard solutions of A.Ch. (10 and 20γ per liter). Experiment XVIII (4/10/1937). Note that the sensitivity of the leech progressively diminished but at the end of the experiment it still was almost 50 per cent of the initial one. Dilution of the perfusate 1:1.4.

table represent only a rough estimate of the amount present. They can, however, be safely considered as indicating the maximal amount of A. Ch. contained in the samples of perfusate.

The most remarkable feature of the observations was the small amount of A. Ch. released by preganglionic stimulation; for instance, in period 6 a tetanus made up of some 600 shocks caused maximal opening of the pupil and retraction of the nictitating membrane, but nevertheless failed to bring enough activity into the perfusate to cause visible movement in the leech muscle. This result was so unexpected that the preparation was immediately calibrated with A. Ch. at 10γ per liter to ascertain whether or not it was still active; and it was found that the sensitivity was only slightly less than during previous applications. If there was any A. Ch. in the sample, its concentration was less than 2γ per liter, i.e. about a hundredth part of the amount obtained in the early experiments. (In

the latter the rate of perfusion had been slower, but a lower frequency of stimulation compensated for the slow rate.) However, control samples 7 and 8 contained small but measurable amounts of A. Ch., indicating that there was some delayed output of the substance.

In the later periods of preganglionic stimulation the amount of A. Ch. obtained was somewhat larger, but still very small. Even when, after some 30 minutes of perfusion, A. Ch. began to appear in appreciable amounts in the control samples, preganglionic and postganglionic stimulations caused only small increases in the yield, and of the amounts produced a large fraction was released in the period following the peripheral responses. At the end of the experiment histological examination showed that this ganglion was in excellent condition.

TABLE 3

Experiment XIX (4/13/1937)

SAMPLE	DURATION OF COLLECTION	AMOUNT OF FLUID	APPROXIMATE CONCENTRATION OF ACETYLCHOLINE	SAMPLE	DURATION OF COLLECTION	AMOUNT OF FLUID	APPROXIMATE CONCENTRATION OF ACETYLCHOLINE
	minutes	*cc.*	*γ per liter*		*minutes*	*cc.*	*γ per liter*
1C	3	2.4	Less than 5	16D	2	1.1	50
2D	2	?	15	17C	2	1.1	15
3D	2	0.8	More than 50	18V	2	Lost	
4C	2	1.1	15	19C	2	1.2	Less than 5
5C	2	1.0	10	20D	2	0.9	More than 50
6D	2	0.7	More than 50	21C	2	1.0	50
7C	2	1.1	20	22C	2	1.0	30
8C	2	1.0	Less than 5	23D	2	0.9	More than 50
9D	2	1.0	More than 50	24C	2	1.0	20
10C	2	1.2	12	25C	2	0.9	10
11C	2	0.8	10	26D	2	0.7	More than 50
12D	2	0.9	More than 50	27C	2	0.9	50
13C	3	1.4	15	28C	2	0.8	15
14C	2	0.9	Less than 5	29D	2	0.8	50
15C	3	1.4	10	30C	2	0.8	6

Experiment XIX (4/13/1937) (fig. 7, table 3). The vaporizer was omitted in this experiment and in its stead the room was maintained at 37 degrees C. and at high humidity. Some difficulty was experienced, however, in keeping the tissues moist under these conditions. A chance observation in the course of the experiment indicated that A. Ch. appears promptly in the perfusing fluid. In period 2, which lasted two minutes, the perfusate of only the last half minute carried the products of activity resulting from preganglionic stimulation. Yet the perfusate was highly active. The appearance of the substance within 30 seconds supports Feldberg and Vartiainen's statement (1934, p. 111) that A. Ch. is removed

from the ganglion by the perfusion fluid in less than one minute after its liberation.

The durations of the preganglionic tetani (10 maximal shocks per second) were: 1 minute in periods 3, 9, 16, and 23; 1 minute and 40 seconds in period 20; and 2 minutes in periods 12, 26, and 29. The samples of perfusate were tested in 1:1.4 dilution.

As shown by the records in figure 7, preganglionic stimulation never failed to cause the release of considerable amounts of A. Ch. But the time of its appearance did not correspond closely with either the period of stimulation or the peripheral response. As usual, the peripheral response started immediately after commencement of stimulation and began to subside as soon as the stimulus was removed, relaxation of the muscles becoming complete, as far as it could be determined by direct observation, within less than a minute. But the release of A. Ch. continued for many

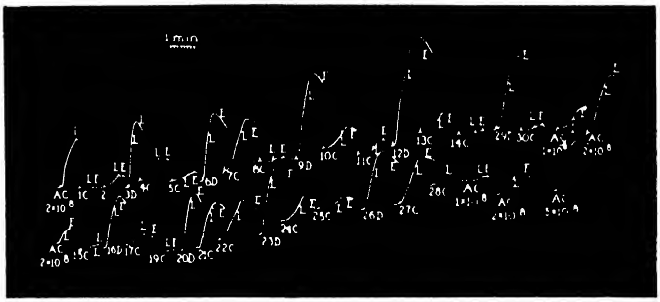

Fig. 7. Responses of the leech to the perfusate and to standard solutions of A.Ch. (10, 20 and 50γ per liter). Experiment XIX (4/13/1937). The sensitivity of the leech muscle was low but remained constant. Dilution of the perfusate 1:1.4.

minutes after the end of stimulation. In some cases the liberation of A. Ch. was found to have ceased (insofar as the rather insensitive leech could detect) when the second control sample after stimulation was taken (4, 10, 17, 24, 30; 13 was not conclusive because one minute was lost between 12 and 13), but in other cases even the second control sample was highly active (7, 21, 22, 27). The prolonged output of A. Ch. could not have been due to slowed diffusion, first because as indicated above, the diffusion of A. Ch. is very rapid, and second because after stimulation period 29, when one should expect the diffusion to be slowest, no prolongation was present. Delayed liberation, which has been noted by Barsoum, Gaddum, and Khayyal (1934) and was seen in every experiment of this series, must mean delayed production.

Between control periods 14 and 15 the perfusion was stopped for 15 minutes, which may be the reason why sample 15 contained more A. Ch. than 14. The asphyxia of the ganglion caused a reduction of the periph-

eral response in period 16 and also a reduction in the liberation of A. Ch. in that period.

The rate of perfusion remained very nearly constant throughout the experiment. During most of the periods of preganglionic stimulation the perfusion rate was reduced, despite the large amount of A. Ch. which was formed.

When the ganglion was examined histologically, it was found to have been greatly damaged by the perfusion, probably because of the drying that had occurred in the course of its preparation. The ganglion itself and the tissues around it were invaded by a large number of white blood

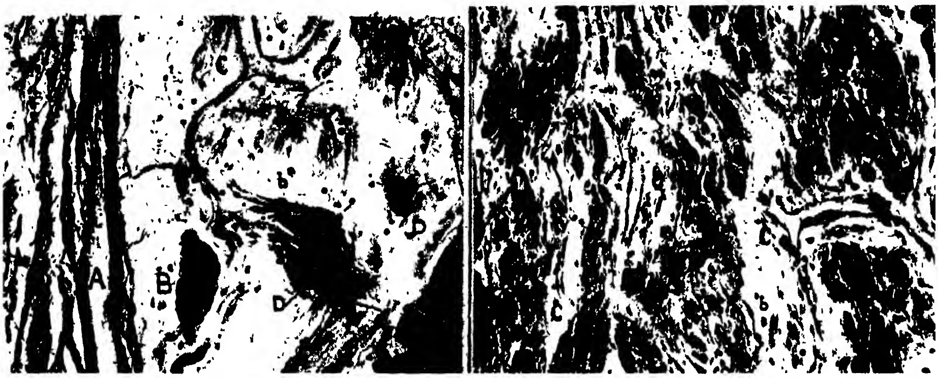

Fig. 8. Sections through the superior cervical sympathetic ganglion perfused in Experiment XIX (4/13/1937), Bodian's silver method, counterstain with acid fuchsin. Magnification 125×. The section on the left is through the periphery of the ganglion; it shows that the capsule of dense connective tissue, *A*, was separated from the ganglion by the edema. In the space thus created there are a capillary, *B*, blocked by a coagulum, an open capillary, *C*, with several branches, islands of ganglion cells, *D*, *D*, and a large number of white blood cells, *b*. The section on the right through the center of the ganglion shows the neuropil broken into small islands of cells by the edema around the capillaries, *C*, *C*, *C*. The ganglion cells are considerably retracted. Numerous white blood cells, *b*, are found in the edematous space.

cells. Edema had distended the dense capsule of connective tissue and created a large space between it and the ganglion proper, in which there were isolated capillaries and fragments of neuropil (fig. 8). The neuropil was generally fragmented and the cell bodies were shrunken and isolated into small patches. In every way the picture was in striking contrast with that of the ganglia in the preceding experiments in which the perfusions had been successful.

Experiment XX (4/16/1937) (fig. 9, table 4). In order more successfully to prevent drying of the tissues, during this experiment the humidity of the room was kept at saturation (37°). Owing to difficulty in making the insertion of the collecting cannula, the first samples were not obtained until 10 minutes after the start of the perfusion. After the eleventh sample,

the cat died, making observations of the responses no longer possible, but not causing interference with the ganglion which was on perfusion. The preganglionic tetanus (shocks more than six times the threshold strength for opening of the pupil, i.e., maximal at the rate of 10 per second) caused in periods 2 and 5 the usual maximal peripheral response; later, of course, no peripheral response could be obtained. The stimulation lasted 1½ minutes in period 2, 2 minutes in 5 and 11, and 3¼ minutes in 20 and 26.

This experiment very nearly reproduced experiment XVIII in that at the beginning of the perfusion the amount of A. Ch. liberated was very

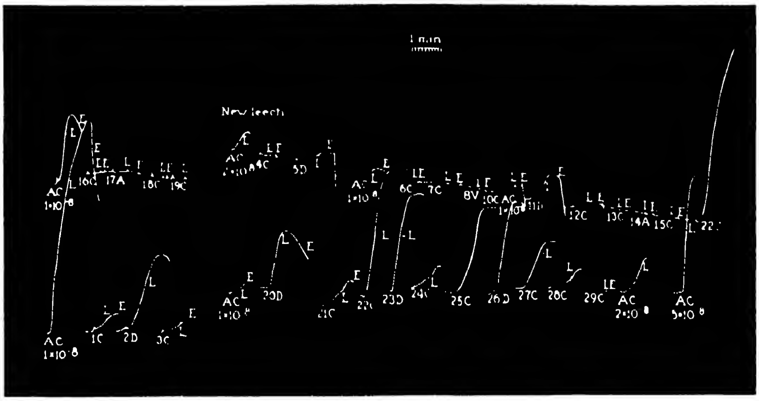

Fig. 9. Responses of the leeches to the perfusate and to standard solutions of A.Ch. (10, 20, and 50γ per liter). Experiment XX (4/16/1937). The responses in the left side were obtained with a leech of high sensitivity; those on the right side with a leech of less sensitivity. While the second leech was being prepared the perfusate was kept in the icebox at 0°C. Dilution of the perfusate 1:1.4. Sample 22 was tested on half of the upper series after further dilution 8:10 with eserinized Locke's solution, more than two hours after the test on the leech strip of the lower series. It showed no loss of activity.

small. The concentration in samples 2 and 5, collected while respectively about 900 and 1200 maximal shocks were being applied, certainly was no higher than 5γ per liter. Thus the amount of A. Ch. per maximal preganglionic volley was much smaller than the amount calculated by Feldberg and Vartiainen (1934). As the perfusion progressed, the A. Ch. production increased, both during periods of rest and of stimulation. It is to be noted that preganglionic tetani 20 and 23 caused liberation of A. Ch. in an initial and a delayed burst, and that after tetanus 26 the release of A. Ch. continued for 8 minutes after the end of the stimulation.

The histological analysis revealed that the greatest part of the ganglion was in apparently normal condition, but in the oral pole there were some distended capillaries surrounded by slightly edematous neuropil.

DISCUSSION. The present study has revealed several significant facts:

1. In perfusions made with a technique likely to cause damage to the ganglia there appeared at the start of the perfusion in the absence of stimulation, a considerable output of A. Ch. (fig. 2, *1 c*). Careful technique considerably reduced or prevented this spontaneous release (figs. 4, 5).

2. In successful perfusions the liberation of A. Ch. by preganglionic stimulation was very small. For instance, the concentration of A. Ch. observed at the beginning of experiments XVIII and XX (fig. 6 and 9) was not greater than 5γ per liter; but when the perfusion was defective, the amounts of A. Ch. released were many times greater, up to 250γ per liter.

TABLE 4

Experiment XX (4/16/1937)

SAMPLE	DURATION OF COLLECTION	AMOUNT OF FLUID	APPROXIMATE CONCENTRATION OF ACETYLCHOLINE	SAMPLE	DURATION OF COLLECTION	AMOUNT OF FLUID	APPROXIMATE CONCENTRATION OF ACETYLCHOLINE
	minutes	*cc.*	*γ per liter*		*minutes*	*cc.*	*γ per liter*
1C	3	1.3	1	16C	3	0.7	Less than 2
2D	2	0.7	5	17A	3	0.7	Less than 2
3C	3	1.1	1	18C	3	0.7	Less than 2
4C	3	1.0	Less than 5	19C	3	0.7	Less than 2
5D	3	0.9	5	20D	4	0.8	30
7C	3	0.9	Less than 5	21C	4	0.8	15
8V	3	0.8	Less than 5	22C	4	0.7	40
9C	3	0.8	Less than 5	23D	4	0.8	40
10C	3	0.8	Less than 5	24C	4	0.8	15
11D	3	0.8	15	25C	4	0.8	20
12C	3	0.8	5	26D	4	0.7	40
13C	3	0.8	Less than 5	27C	4	0.8	30
14A	3	0.8	Less than 5	28C	4	0.9	10
15C	3	0.7	Less than 5	29C	4	0.8	Less than 5

3. In the more complete experiments of the fourth series, in which histological analysis failed to reveal signs of damage to the ganglia, a definite relation between the output of A. Ch. and the duration of perfusion was established. At the start of the experiments the amounts of A. Ch. liberated were small, the minimal amount being produced in the second period of stimulation (figs. 5, *5 A*; 6, *6 D*; 9, *5 D*). The amounts increased during the perfusion and decreased again toward the end of the experiment. The increase in the release of A. Ch. was accompanied by a decrease in the rate of perfusion.

These facts raise the question as to whether the liberation of A. Ch. by nerve stimulation is a physiological process or, as recently Fleisch, Sibul, and Kaelin (1936) have concluded for motor nerves to striated muscles, a pathological process.

There can hardly be any doubt that the large outputs of A. Ch. observed

at the start of perfusion in the absence of stimulation, as well as similar outputs reported by Brown and Feldberg (1936 a, b), were attributable to injury of the tissues, for these amounts were prevented by improvements in the technique. Likewise the large outputs of A. Ch. observed after preganglionic stimulation in a number of the present experiments, as well as in the experiments reported by Feldberg and Gaddum (1934), Feldberg and Vartiainen (1934), Barsoum, Gaddum and Khayyal (1935), and Brown and Feldberg (1936 a, b), also in blood-perfused ganglia[2] must be considered as being pathological for the following reasons:

1. Physiological transmission through the ganglion takes place with release of much smaller amounts of A. Ch.

2. The amounts of A. Ch. liberated increase during the course of the experiment, as the rate of perfusion drops.

3. Ganglia releasing A. Ch. in large amounts are shown by histological analysis to have suffered heavy damage.

Taking the experiments as a whole, it must be concluded that the release of A. Ch. is favored by damage of the tissues. It also seems likely that some A. Ch. is released by dying cells, because in the experiments of the first and second series it was noticed that after a large spontaneous output of A. Ch. had taken place, further stimulations were ineffective in releasing the substance.

However, the fact that A. Ch. is released in any amount must indicate that the substance is found to some extent in the normal metabolism of the tissues of the ganglia. Whether or not the substance would diffuse through the surface of the cells under physiological conditions cannot be stated. At any rate, a diffusion such as this cannot take place to an extent greater than is observed in the early part of the most successful perfusion experiments (exps. XVIII and XX).

The metabolism of A. Ch. following nerve stimulation cannot be considered as a specific synaptic process, because A. Ch. may be released in considerable amounts by the passage of impulses through the nodosum ganglion of the vagus and by antidromic conduction of impulses into the ganglion cells. Furthermore, the liberation of A. Ch. usually outlasts the conduction of impulses and it may begin some time after transmission has ceased. Delayed production of A. Ch. cannot be doubted. The only question is whether there is any immediate production. Unfortunately, the technique of perfusion does not permit us to measure the time of liberation of A. Ch. within less than 30 seconds, while the synaptic

[2] In the opinion of the present author, satisfactory perfusion with blood of the isolated superior cervical and nodosum ganglia is not feasible. The operation would have to be performed as indicated in the fourth series of experiments, and the coagulation of the blood would have to be absolutely prevented. It appears that the prevention of coagulation is incompatible with a normal circulation and normal oxygenation of blood in the lungs; at least this was found to be the case with the anticoagulants employed (Chlorazol fast pink, Novirudin, Liquoid).

delay in sympathetic ganglia lasts for about 0.002 second (Brown, 1934; Eccles, 1933, 1936).

Examination of the records presented in figures 5, 6 and 9, which are those of the three best experiments, reveals that in general at the starts of the perfusions, preganglionic stimulation (fig. 6, *2 D*, *10 D*; fig. 9, *2 D*, *5 D*, *11 D*) caused an immediate output of A. Ch., while under similar conditions antidromic stimulation (fig. 5, *4 C*, *6 C*, *9 C*) seemed to cause only a delayed output. This might be considered as an indication that preganglionic stimulation causes release of A. Ch. during transmission, while ganglion cells liberate the substance after conduction. But the experiments also show that antidromic stimulation may cause an immediate output of A. Ch. (fig. 1, *5 A*, *7 A*; fig. 5, *11 A*), and that preganglionic stimulation may fail to cause an immediate output, while initiating a delayed one (fig. 6, *7 C*, *8 C*, *9 C*); or it may cause a delayed output (fig. 9, *22 C*) larger than the initial one (*20 D*). The similarity between records *20*, *21*, *22* and *23*, *24*, *25* in figure 9, and records *11*, *12* and *13* in figure 5 indicates that in both cases the same mechanism was being activated. Also, stimulation of the vagus may cause an immediate (fig. 3) or a delayed output (fig. 4), and in this case there can be no doubt that the A. Ch. is always released by the same elements.

Thus there is no evidence to show that preganglionic stimulation causes the production of A. Ch. by activating a mechanism fundamentally different from that activated by impulses started in the postganglionic trunk or in the vagus nerve; although it must be emphasized that preganglionic stimulation seems to cause an immediate output of A. Ch. more readily than any other stimulation. Thus far no satisfactory explanation has been found, either for the variability of the amount liberated or for the delayed output of the substance, but there are two facts which might lead to an understanding of the problem:

1. Postganglionic stimulation reaches only a fraction of the ganglion (the cells of origin of the carotid nerve), while preganglionic stimulation reaches, through the preganglionic arborizations, every cell of the ganglion. Evidently, if the concentration of A. Ch. released by preganglionic stimulation from ganglia in good condition scarcely reaches threshold for the leech, the concentration produced during postganglionic stimulation must remain below threshold.

2. Damaged ganglia release many times more A. Ch. than do ganglia in good condition. Therefore, if during the perfusion a few cells are damaged, they will release larger amounts of A. Ch. than the rest of the ganglion. In fact it is very likely that even in the best perfusions some of the A. Ch. liberated was released by damaged elements. Usually the first period of stimulation (figs. 5, *4 C*; 6, *2 D*; 9, *2 D*) caused a larger output than the second one (figs. 5, *6 D*; 6, *6 D*; 9, *5 D*); and no other explanation

for it can be found than the release of large amounts of the substance by a few cells damaged during the operation.

SUMMARY

1. The liberation of A. Ch. by the superior cervical sympathetic ganglion and the nodosum ganglion of the vagus has been studied with the technique of perfusion described by Kibjakow.

2. Ganglia prepared with a technique leading to damage of the cells as revealed by subsequent histological analysis yield A. Ch. to a perfusate without the intervention of stimulation; and stimulation of either the preganglionic or postganglionic trunks of the sympathetic or of the vagus nerve, causes the liberation of A. Ch. in large amounts.

3. Ganglia prepared in a way so that no damage is revealed on histological analysis do not yield A. Ch. spontaneously and the amounts released as the result of stimulation are very small. At the start of successful perfusions even perfusates obtained after tetanization of the preganglionic trunk with maximal shocks contain A. Ch. in amounts scarcely detectable by the leech preparation.

4. Release of A. Ch. regularly takes place some time after the transmission of impulses has ceased; whether there is any liberation preceding synaptic transmission is still an open question.

5. Since damage of the tissues favors the release of A. Ch. and perfusion of the ganglia always creates abnormal conditions, it cannot be stated whether physiologically any amount of A. Ch. diffuses out of the cells; but the evidence indicates the existence of an A. Ch. metabolism in the tissues of the ganglia.

6. The A. Ch. metabolism is not a process which is specific to the synaptic junctions.

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DIFFERENTIAL AMPLIFIER*

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Two systems of differential amplifiers have recently been published in this journal by Offner[1] and Schmitt.[2] The latter has named six defects which must be avoided in differential input action. To these it is desirable to add three other conditions. The accuracy of the differential formation should not be affected:

1. By changes in the operating voltages.
2. By variations in the amplification factors of all the tubes taking part in the differential formation.
3. With high amplitudes of the modulating voltages common to both input leads.

It is desirable to satisfy conditions 1 and 2 in all applications of differential amplifiers, while regard for condition 3 is important in some technical applications, and especially in the high gain amplifiers used in biological work where there are often electrode contact potentials of large value upon which are superimposed the relatively small potential changes that it is desired to observe.

The differential circuit shown in Fig. 1 avoids to a large extent the possibilities for defects that have been mentioned. The differential action is accurate to better than 1 part in 1000 for common input voltages up to 20 volts. In common with the circuit of Schmitt or Matthews,[3] this amplifier possesses the advantage that after the differential input stage only single tube following stages are needed.

In the circuit of Fig. 1 the voltage change E_1 between electrode *a* and ground is led to the grid of tube 1 and is amplified across R_p. At

* A more extended paper with the circuit of Fig. 1 was first submitted for publication to the editors of this journal on July 20, 1936.

[1] Franklin Offner, Rev. Sci. Inst. **8**, 20 (1937).

[2] Otto H. Schmitt, Rev. Sci. Inst. **8**, 126 (1937).

[3] B. H. C. Matthews, J. Physiol. **81**, 28 Proc. (1934).

the same time, change E_2 between ground and the electrode b is carried to the grid of tube 2, which has a plate circuit resistance only between the cathode and a negative part (−90) of the plate battery. Therefore, all the modulation of grid 2 is effective across the resistance R_c, and since this modulation backfeeds to the grid cathode difference,

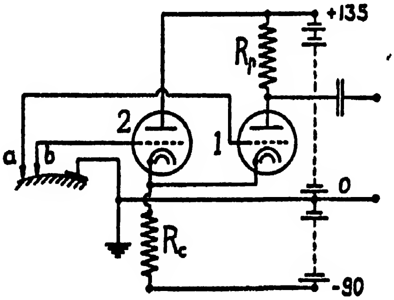

FIG. 1. Fundamental circuit

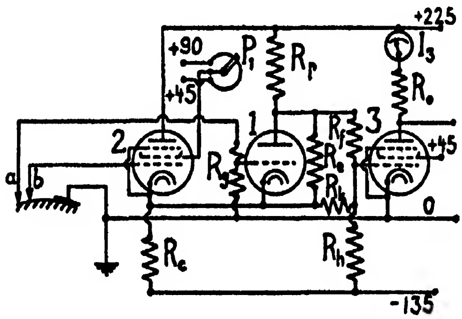

FIG. 2. Circuit of a d.c. coupled input stage with adjustments of the differential formation

tubes 1, 2, and 3: RCA 1603 R_f = 300,000 ohms
R_c = 100,000 ohms R_h = 600,000 ohms
R_p = R_o = 200,000 ohms P_1 = 30,000 ohms.

For adjusting the differential formation there might be used alternatively:
R_g divided in a ratio: ~1:100
or R_s: ~500,000 ohms
or R_h: ~2 megohms.

the modulation on $R_c = \Delta I_2 \cdot R_c$, which is equal to the modulation of the cathode in relation to ground, will be:

$$\Delta I_2 \cdot R_c = E_2(G_2/G_2 + 1). \qquad (1)$$

G_2 is the gain of tube 2 in the usual arrangement.

The higher G_2 is, the more will the factor $G_2/G_2 + 1$ approach to-

wards 1, but it never reaches 1. The cathode of tube 1 is connected with the cathode of tube 2 and thus receives the same modulation as the cathode of tube 2. Tube 1 is modulated on the grid with the changes E_1 on electrode a, and on the cathode with the changes E_2 on electrode b. The differential formation of the amplifier will be correct when an equal modulation of the grids of tubes 1 and 2 ($E_1 = E_2$) does not produce any potential changes on the plate of tube 1. The condition is I_1 = constant.

Actually tube 1 is modulated in three ways: (a) on the grid $= E_1$; (b) on the cathode against the grid $= E_2(G_2/G_2 + 1)$ (1); and (c) on the cathode against the plate also with the amount of $E_2(G_2/G_2 + 1)$. For the purpose of calculation this value must be reduced to a corresponding grid-cathode modulation and for I_1 = const. this will be $E_2(G_2/G_2 + 1)\cdot(1/\mu_1)$, when μ_1 is the theoretical amplification factor of tube 1.

The fact that the current I_1 flows through R_c to the negative part of the plate battery does not affect the degree of modulation of the cathodes (b), because the resistance of tube 1 equals ∞ for the condition I_1 = constant.

All three modulations should compensate one another:

$$E_1 - E_2\frac{G_2}{G_2 + 1} - E_2\left(\frac{G_2}{G_2 + 1}\right)\frac{1}{\mu_1} = 0, \tag{2}$$

$$\frac{\mu_1 - G_2}{(G_2 + 1)\cdot\mu_1}\cdot E_1 = 0; \qquad (E_1 = E_2).$$

This equation is correct for $\mu_1 = G_2$. By the use of the same type of tubes for tube 1 and tube 2, G_2 will always be smaller than μ_1 and this produces a small error in the differential formation. The error x will be:

$$x = \frac{\mu_1 - G_2}{(G_2 + 1)\mu_1}\cdot E_1. \tag{3}$$

Tube 1603 (antimicrophonic type) operated as triode with the resistance values listed for Fig. 2 has $\mu_1 = 20$ and $G_2 = 16$; $x = 4/340 = 1.17$ percent. This small value can be adjusted to 0 in different ways: By use of a grid leak resistance for tube 1, the grid could be connected on a tap of it (ratio 1.17 : 98.83).

For biological recording often any grid leak resistance is undesirable, and in cases such as these the resistance R_e may be connected across tube 1. It couples a small amount of the cathode modulation directly to the plate of tube 1, which before that could not completely balance the modulation of grid 1. When, as shown in Fig. 2, the following normally operated stage (tube 3) is d.c. coupled to the differential input stage by means of a bleeder (R_f and R_h), the resistance R_k may be placed between the cathodes of tubes 1 and 2 and the grid of tube 3.

Another way of correcting the small error would be the use of a steeper type of tube in place of tube 2. Thus the error might be reversed and adjusted to 0 by means of a small variable resistance between the plate of tube 2 and the positive plate voltage. Since the gain of a tube slightly increases with the plate voltage, tube 2 can be made sufficiently steep by using a higher negative plate voltage and a correspondingly higher value for R_c.

For n percent change of G_2 or μ_1 the error in the differential formation would be according to Eq. (3) approximately n/G_2 percent or n/μ_1 in contrast to other differential amplifier circuits, where the error would be n percent. When in a phase reversing differential circuit, a common modulation M has carried the operating point of the tubes to a point where the gain is n percent different, the error occurs with n percent. In this circuit the actual modulation between grid and cathode amounts, due to the backfeeding for tube 2, and the two-side modulation of tube 1, only to the value of the common modulation divided by G_2 or μ_1. Therefore, the common modulation may be G_2 times as large as the usual length of the grid voltage characteristic and n percent change of G_2 or μ_1 would appear only after a common modulation of $M \cdot G_2$ or $M \cdot \mu_1$ has been applied. While n percent change of G_2 produces only n/G_2 percent error in the differential formation, the common modulation in this circuit must be $M \cdot G_2^2$ or $M \cdot \mu_1^2$ before an error of n percent can enter into the differential formation. For $G_2 = 15$ the accuracy is about 225 times higher.

Tube 1 needs less grid bias than tube 2, which operates on an over all higher plate voltage. This can be compensated by connecting the plate of tube 2 to a lower point of the positive plate battery (e.g. + 90 volts). For a d.c. coupled amplifier it is convenient to operate tube 2 as a pentode with a screen grid voltage controlled by a potentiometer

P_1. The gain of tube 2 is nearly unchanged, because both plate and screen grid have fixed voltages.

In order to reach a higher effective amplification of the input stage, tube 2 may also be operated as a pentode. The screen grid voltage may be taken from a tap of the grounded plate voltages. Due to the screen grid current across R_o, the accuracy is somewhat diminished, but is still much higher than in any other differential amplifier circuit.

In triode operation the error can easily be adjusted for 0.02 percent for a working range of 100 mv or more, which covers most biological applications: and without any checking of the differential formation, the amplifier will be safe within 0.1 per cent over periods of months for all customary changes in supply voltages and tube characteristics. With a proper arrangement of the amplifier parts, these values can be effective over a frequency range from d.c. amplification up to 15,000 cycles (limit of the tests made).

The accuracy to which the differential formation can be adjusted is limited merely by factors of unstability in the tubes such as backheating of the cathodes by the plate current or by small changes in the B-batteries resulting from a big modulation. By replacing any one of the tubes in use by another one of the same type without readjustment of the differential formation only in one case out of ten an error of 1 in 500 entered. The common modulation was in these tests 1 volt. A change of the heating voltage from 6 volts to 4 volts produced an error of less than 1 in 2000. A common change of all plate voltages for ±10 percent gave a similar error. If only one part of the plate current supply was varied for ±10 percent the error is within 1 percent, but such changes exceed the limits of usual working conditions even in power supply operated setups. The amplification factor itself will undergo of course much larger variations when the described changes happen to occur.

SUMMARY

An amplifier input circuit, which differentiates with a high degree of dependability a modulation between two ungrounded points against the common modulation of these points, has been described. After this input stage, normally operated stages, grounded on one side, can be used.

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POTENTIALS IN HALICYSTIS AS AFFECTED BY NON-ELECTROLYTES

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In diluting the sea water surrounding marine algae it is desirable to add something to maintain the osmotic pressure, otherwise the cells take up water and may burst. Damon[1] used glycerol for this purpose in experiments on *Valonia*. He found that the changes in P.D. at 20°C. obeyed the equation

$$\text{P.D.} = 58\,\frac{V_{Cl} - U_{Na}}{V_{Cl} + U_{Na}}\log\frac{C_1}{C_2}$$

where V_{Cl} and U_{Na} are the apparent mobilities of Cl^- and Na^+ in the outer protoplasmic surface, C_1 is the higher and C_2 the lower concentration of sea water. Putting $V_{Cl} = 1$ he obtained 0.2 for the value of U_{Na} (on the assumption that $C_1 \div C_2$ was the same in the protoplasmic surface as in the external solution).

The limiting value of the P.D. in this case may be obtained by putting $U_{Na} = 0$. We then obtain 17.4 mv. as the limit when $C_1 \div C_2 = 2$. This value is a little less when we employ activities in place of concentrations.[2]

Similar experiments with *Halicystis*[3] yield a different result. In view of the work of Blinks[4] it was decided to add sufficient $CaCl_2$ and KCl to the glycerol solution to make the concentration approximately the same as in sea water. The solution contained 1.1 M glycerol + 0.02M $CaCl_2$ + 0.012 M KCl.

When sea water is diluted with an equal volume of this solution (at pH 8.2) and is then substituted for natural sea water (at pH 8.2) we find that in one minute[5] or less the P.D. becomes about 29 mv. less positive.[6] Since this value is in excess of the theoretical limit of 17.4 mv. it seems possible that glycerol has altered the value of $C_1 \div C_2$

in the protoplasmic surface so that instead of being 2, as assumed, it has reached some higher value.

This might happen as follows. If the partition coefficient S (conc. of electrolyte in protoplasmic surface ÷ conc. of electrolyte in external solution) were constant, the value of $C_1 \div C_2$ would be the same as in the external solutions. But if S in the dilute sea water is lessened by the glycerol, the value of C_2 will be correspondingly small and that of $C_1 \div C_2$ will be greater.

It seems advisable, for the present at least, to employ the term partition coefficient in a very broad sense since, for example, the effect of NaCl may depend not only on the number of sodium ions in the protoplasmic surface but also on complexes in the sense of Kraus,[7] e.g. $(NaX_{I})^+$ and $(NaX_{II})^+$ where X is an element or a radical. All such complexes in the protoplasmic surface plus the actual sodium ions may be lumped together and their sum in unit volume of the non-aqueous surface layer divided by the sum of the potassium ions in unit volume of the external solution may be called the "apparent partition coefficient."

A variety of other explanations is possible, such as the following:

(1) Mechanical rupture of the outer protoplasmic surface layer, the process being fully reversible. If this layer is liquid, as seems possible, this process might take place as in (unpublished) experiments with oily films spread out on the surface of aqueous solutions when chemical action is going on. In such cases breaks in the film may appear and disappear as the result of local action. A great many are usually present and the amount of short-circuiting and consequent loss of P.D. might change gradually, depending on the number of breaks in the film.

This process might cause a total loss of P.D. at either protoplasmic surface. If it occurred at one surface while the other had a negative P.D. the resultant P.D.[8] would be negative.

(2) Production of organic ions in the protoplasm which lessen the outwardly directed potential.

(3) Changes in potentials not due to diffusion, e.g., in phase boundary potential or in membrane potential.[9]

Glucose, sucrose and maltose act like glycerol, as shown in table 1. One striking effect is common to all of these, namely, the recovery of

the original P.D. on standing.[10] For example, when sea water is replaced by sea water plus an equal volume of isotonic glycerol solution (containing $CaCl_2$ and KCl) the P.D. becomes less positive in the course of about 20 seconds to the extent of about 29 mv. But a few seconds later the P.D. begins to grow more positive again[11] as though the non-electrolyte were penetrating to the inner protoplasmic surface and there setting up changes opposite in sign to those produced at the outer surface. Such changes at the two surfaces might very well be opposite in sign since the surfaces are known to differ greatly.[12]

TABLE 1

Change of P.D. Produced by Replacing Sea Water in Contact with Halicystis by Sea Water Plus an Equal Volume of an Isotonic Solution Containing 1.1 M Non-Electrolyte + 0.02 M $CaCl_2$ + 0.012 M KCl. All at pH 8.2 Unless Otherwise Stated*

Non-electrolyte in substituted solution	Change in P.D.	Number of observations†
Glycerol	28.9 ± 0.83	5
Glucose	27.8 ± 4.74	6
Sucrose	17.1 ± 1.56	6
Maltose	38.0 ± 1.59	13
Mannite	13.8 ± 0.52	14
Mannite pH 6.4	9.8 ± 0.68	13

* In each case the P.D. became less positive (the sign is positive when the positive current tends to flow outward from the sap to the external solution).

† A limited number of cells was available.

They would be possible even if the surfaces were alike provided ions produced in the protoplasm diffused inward as well as outward.

In the course of 5 minutes or less the P.D. usually returns approximately to the original value in sea water. If the cell is then transferred to sea water little or no change in P.D. occurs.[13] This may mean that the glycerol is washed out of both surfaces at about the same rate so that the changes in P.D. at one surface are cancelled by those at the other.

A different picture is presented when we employ mannite.

(1) With mannite the theoretical limit is not as a rule exceeded. The average loss of P.D. when sea water is replaced by sea water plus

an equal volume of 1.1 M mannite + 0.02 M $CaCl_2$ + 0.012 M KCl at pH 8.2 is about 14 mv. An occasional measurement runs higher[14] (up to 22 mv.). Experiments at pH 6.4 gave a lower value (table 1). These were made by lowering the pH of the sea water from 8.2 to 6.4 and then transferring to the dilute sea water at 6.4. The low value may be connected with the fact that the P.D. is reduced by lowering the pH of the sea water (this will be discussed in a subsequent paper).

(2) The P.D. thus produced remains constant for 8 minutes or more. It does not as a rule increase again and show "recovery."[15]

In view of this it would seem advisable to use mannite in studying the concentration effect. We cannot be sure that it does not affect the values obtained but it appears to do so less than the other substances mentioned.

The high value obtained for the concentration effect with mannite is of interest. If we use this value to calculate $U_{Na} \div V_{Cl}$ we obtain a value very much less than that found for *Valonia*[1] (in *Valonia* $U_{Na} \div V_{Cl} = 0.2$).

To account for the values in table 1 in excess of the theoretical, the simplest assumption seems to be that partition coefficients are altered by all the non-electrolytes except mannite. Unfortunately the theory[16] of partition coefficients is not yet developed and at present we can do little more than record suggestive facts. If non-electrolytes can alter the partition coefficients of electrolytes it is evident that this may be of importance for the cell.

It would seem that if glycerol lessens the partition coefficients of electrolytes, as suggested, the addition of glycerol to sea water should make the P.D. less positive. This is the case. Enough glycerol was added to sea water to increase the osmotic pressure by about 50 per cent. Enough solid NaCl was then added to bring the halide content up to the normal (0.58 M). In the course of 3 to 5 minutes the P.D. became less positive by 10 to 20 mv. On replacing in sea water the P.D. returned to normal in 4 minutes or less, but in the meantime it became temporarily still less positive to the extent of 5 to 10 mv.

Enough mannite was added to sea water to raise its osmotic pressure about 50 per cent and solid NaCl was then added to make the halide content 0.58 M as usual. Since this produced no change in P.D. we might conclude that, as expected, mannite does not change

the partition coefficient as glycerol does. But mannite is not wholly without effect on the P.D. for when the cells are replaced in sea water the P.D. becomes temporarily less positive to the extent of 10 to 15 mv. After this it returns to normal (the whole process takes about 4 minutes). The cause of these changes is presumably osmotic and is due to the taking up of water by the different parts of the protoplasm. As might be expected this is similar with glycerol and with mannite.

That the taking up of water can cause the P.D. to become less positive is suggested by experiments with sea water plus an equal volume of distilled water. In this the P.D. became within 15 seconds 44 ± 3 mv. less positive (10 observations).[17] Similar experiments performed by L. R. Blinks yielded smaller values.[18]

Replaced in sea water the cells returned in 4 minutes or less to the normal P.D.[19] In this case we appear to have an effect due to change of electrolyte concentration plus an effect due to the redistribution of water. The effect is therefore much greater than that produced by mannite.

Summary.—To study changes in P.D. caused by diluting the sea water bathing cells of *Halicystis* it is desirable to add a non-electrolyte to maintain the osmotic pressure of the external solution. For this purpose mannite appears to be one of the most suitable. With glycerol, glucose, sucrose and maltose the changes of P.D. are so large that a reversible alteration of the protoplasmic surface is indicated. This may affect the P.D. by changing the partition coefficients of electrolytes or in other ways.

[1] Damon, E. B., *Jour. Gen. Physiol.*, **13**, 445 (1929–30).

[2] Osterhout, W. J. V., *Ibid.*, **13**, 715 (1929–30).

[3] The experiments were done with *Halicystis Osterhoutii* (Blinks, L. R., and Blinks, A. H., *Bull. Torrey Bot. Club*, **57**, 389 (1931)), using the technique described in a previous paper (Osterhout, W. J. V., *Jour. Gen. Physiol.*, **20**, 13 (1936–37)). Temperature about 22°C.

Unless otherwise stated there was no appearance of injury during the treatment or in the following days.

[4] Blinks, L. R., *Jour. Gen. Physiol.*, **13**, 223 (1929–30); **18**, 409 (1934–35).

[5] There is a latent period of 15 seconds or less. This is very variable and may depend to some extent on the thickness and cutinization of the cellulose wall and on

bacterial jelly covering the cell. Such jelly gives the cells a slippery feeling There seemed to be no jelly on these cells.

This latent period was also observed in experiments with KCl, NH_4Cl at pH 8.2 (0.005 M but not with 0.3 M), and 0.01 M guaiacol.

[6] See table 1. A similar result was previously obtained by L. R. Blinks (personal communication).

[7] Cf. Osterhout, W. J. V., *Jour. Gen. Physiol.*, **20**, 13 (1936–37).

[8] For reversal of sign of *Halicystis ovalis* in unbalanced NaCl see Blinks, L. R., Rhodes, R. D., and McCallum, G. A., *Proc. Nat. Acad. Sci.*, **21**, 123 (1935). For reversal in *Valonia* caused by dilute sea water see Damon, E. B., and Osterhout, W. J. V., *Jour. Gen. Physiol.*, **13**, 457 (1929–30).

[9] Cf. Teorell, T., *Proc. Soc. Exp. Biol. Med.*, **33**, 282 (1935), Meyer, K., and Sievers, J.-F., *Helv. Chim. Acta*, **19**, 987 (1936).

[10] This has been observed with glycerol by L. R. Blinks (personal communication).

[11] This does not happen in *Valonia.*

[12] Cf. Blinks, L. R., *Jour. Gen. Physiol.*, **13**, 223 (1929–30); **18**, 409 (1934–35).

[13] If the cell is transferred to sea water when the P.D. is at the minimum (i.e., before "recovery" has started) the P.D. becomes temporarily less positive to the extent of 4 mv. or less; this does not last more than a minute and the P.D. then returns to the normal value. This also applies to mannite. Apparently it does not apply to glucose. Presumably this means a different behavior at the inner and outer protoplasmic surfaces.

[14] Blinks found that a lack of balance in the external solution made the P.D. less positive or even strongly negative. This may play a rôle here.

Cells injured by exposure to a temperature below 14°C. showed no change in P.D. when exposed to sea water plus an equal volume of mannite solution but when glycerol was used in place of mannite they showed the expected change. In both cases the cells were dead the next day. A few lots of cells showed no change in P.D. when transferred to sea water plus an equal volume of mannite solution even though they seemed normal in every other respect. One such lot when tested two days later gave the usual response.

[15] An occasional cell shows a small decrease. Such cells become temporarily less positive when returned to sea water, as in the case of cells exposed to glycerol, but this temporary change is much less than with glycerol.

[16] Cf. Shedlovsky, T., and Uhlig, H. H., *Jour. Gen. Physiol.*, **17**, 549, 563 (1933–34); Falkenhagen, H., *Electrolytes*, Oxford, Clarendon Press, 1934.

[17] If left in the solution 30 seconds or more the P.D. began to increase (as with glycerol) but in most cases the cell was returned to sea water after 20 seconds.

[18] Personal communication.

[19] After the cell was replaced in sea water the P.D. did not become temporarily less positive as in the case of mannite and glycerol.

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CALCULATIONS OF BIOELECTRIC POTENTIALS

II. THE CONCENTRATION POTENTIAL OF KCl IN NITELLA

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In earlier studies[1] the electrical behavior of *Nitella* conformed to the equations of Nernst and of Henderson but recently, in a different set of cells,[2] we have found some apparent exceptions.

These exceptions are illustrated in Fig. 1. The theoretical curve shows the approximate change in P.D. which KCl would produce if the values were due entirely to diffusion potential in the protoplasmic surface[3] and the mobility of K^+ greatly exceeded that of Cl^-. The values were calculated from the equation

$$\text{Change of P.D.} = 58 \frac{U - V}{U + V} \log \frac{C_1}{C_2}$$

where U and V are the mobilities of K^+ and Cl^- respectively, C_1 and C_2 are the concentrations,[4] and V is taken as zero. All these values relate to the non-aqueous surface layer of the protoplasm.

It is evident that the slopes of the observed curves do not surpass that of the theoretical curve except at certain concentrations. Thus when 0.001 M KCl is substituted for 0.000316 M the curve rises abruptly so that its slope exceeds the theoretical.

[1] Osterhout, W. J. V., *J. Gen. Physiol.*, 1929–30, **13**, 715.

[2] The earlier cells (which came from a different locality and will be called Lot A to distinguish them from the present cells which will be called Lot B) showed a lower concentration effect of NaCl and much less inhibitory effect of calcium on the production of negativity by KCl. This will be discussed elsewhere.

[3] It is assumed that the concentration of KCl in the protoplasmic surface is directly proportional to that in the external solution.

[4] Concentrations are employed for convenience in place of activities. If this were not done the straight line would become somewhat curved.

The reason for this abrupt rise is evident when we examine the photographic record shown in Fig. 2.

The observations were made on *Nitella flexilis*, Ag. and were recorded photographically.

Short period recording devices require for their operation either high voltage,

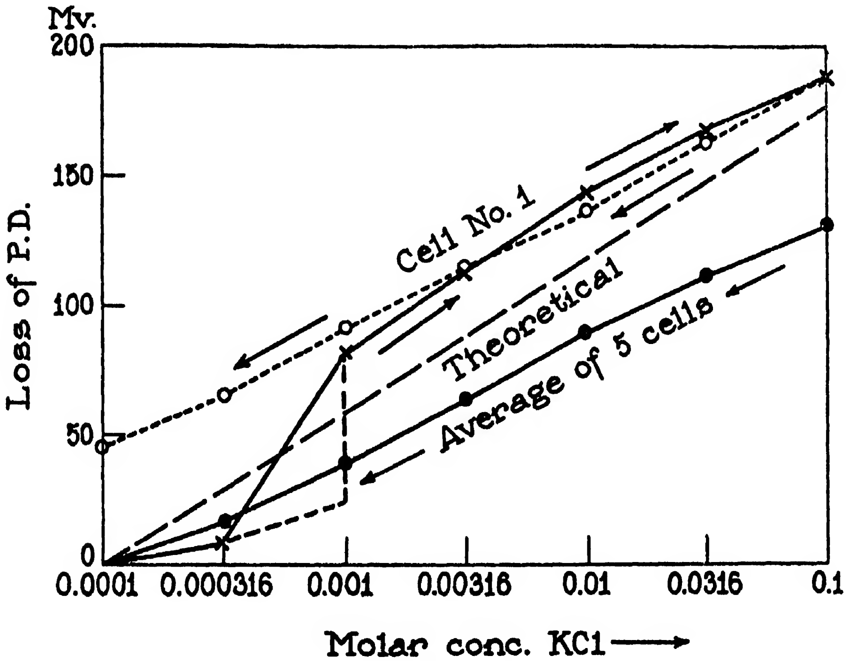

FIG. 1. Effects of KCl on P.D. The broken straight line approximates the theoretical slope of the curve showing change of diffusion potential when the concentration of KCl in contact with *Nitella* increases and the mobility of K^+ greatly exceeds that of Cl^-, partition coefficients being constant. The scale of abscissae is logarithmic: each step is made by multiplying by 3.16 ($= 10^{0.5}$).

The curve with crosses shows measurements on a single cell as the concentration of KCl increases (arrows pointing upward). The curve with open circles (dotted line) shows measurements on the same cell as the concentration of KCl decreases (arrows pointing downward). The lowest curve shows the average of five cells as the concentration of KCl decreases (see p. 222).

The slopes of the curves do not exceed the theoretical except in the curve with crosses where 0.000316 M KCl is replaced by 0.001 M KCl. At this point the change in P.D. occurs in two steps as indicated by the broken line. The first step does not exceed the theoretical; the second is larger and is due to an action current which permanently raises the level of the curve (see Fig. 2).

Temperature 20–21°C.

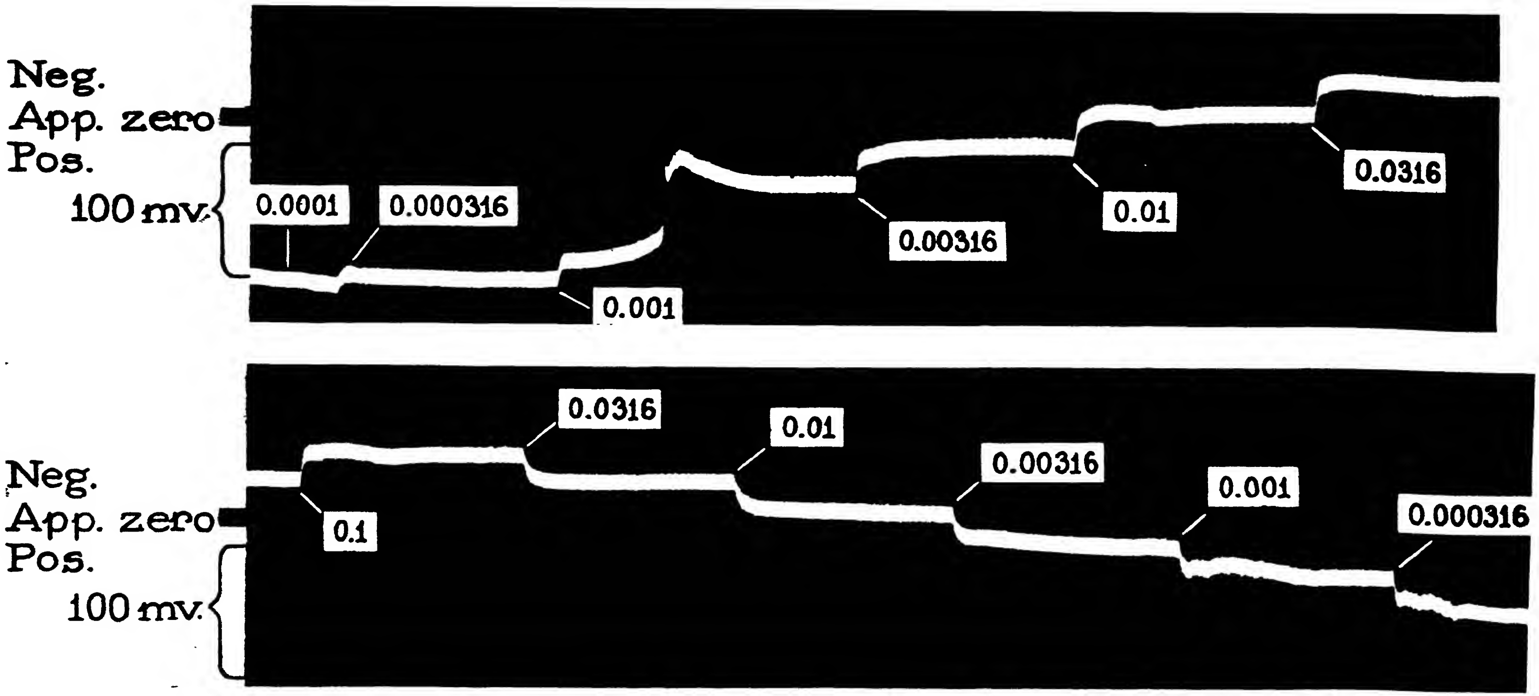

FIG. 2. Photographic record showing changes in P.D. produced by KCl. Three leads were arranged as shown in Fig. 3. The changes of solution were made only at D (the records of C and E, which were in contact with pond water, are omitted to save space). The P.D. at F (which was in contact with 0.01 M KCl) remained constant. It is assumed that its P.D. was approximately at zero, as is usually the case, and hence the label "App. zero" at the left (Cell No. 1 of Fig. 1).

At the start, D was in contact with 0.0001 M KCl and (reckoning from the App. zero) had a positive P.D. of 125 mv. When this was replaced by 0.000316 M KCl the curve rose 7 mv. When this was replaced by 0.001 M KCl the curve rose 15 mv. and then an action current occurred which permanently raised the level of the curve so that on returning to 0.000316 M its level was higher than before (*cf.* Fig. 1).

Heavy time marks 5 seconds apart.

large current, or both. *Nitella* can furnish neither. A vacuum tube amplifier is therefore necessary.

The string galvanometer has adequate speed for *Nitella* and is used because of its simplicity. Tungsten wire replaces the quartz string, as a single tube amplifier is adequate and string breakage is eliminated. (The same amplifier may be used with a quartz string galvanometer by using a 20,000 ohm string shunt. Greater sensitivity and quicker period will result.)

The amplifier shown in Fig. 4 is designed for a galvanometer with tungsten wire in place of quartz fibre, and is grounded at B, the galvanometer wire being 90 volts above ground. The amplifier grounded at B should not be used with a quartz string unless the frame of the galvanometer is connected to the end

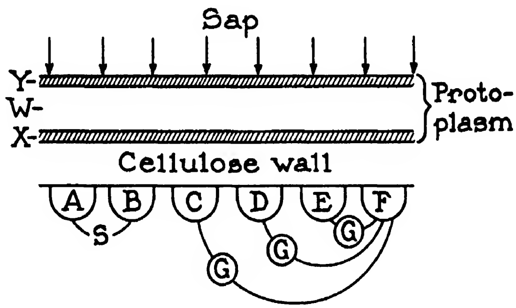

FIG. 3. Diagram to show the arrangement of leads and the supposed structure of the protoplasm which is assumed to consist of an aqueous layer W, an outer non-aqueous layer X, and an inner non-aqueous layer Y.

The arrows show the outwardly directed (positive) P.D. whose seat is supposed to be chiefly at Y when the cell is in pond water: hence the P.D. at X is regarded as negligible and is not shown. But under some conditions the P.D. at X may become important.

Each lead is connected to a separate amplifier and to one string of the 3-string Einthoven galvanometer.

of the string which goes to the slider of P_3. If a high potential is applied between a quartz string and the frame of the instrument, the string will be attracted to the frame and the coating destroyed. This amplifier may be used with 10^6 ohms in the input circuit with little disturbance from A. C. lines, and if the *Nitella* cell and electrodes are placed in a shielded cage, its resistance may be as great as 10^7 ohms with little error. The vacuum tube is operated at its "free" grid potential in order to keep grid current at a minimum.

The function of the amplifier is to furnish current to the string galvanometer. With the circuit constants shown, for each volt change in grid potential there will be a change of 1500 microamperes in current through the galvanometer string. The linear range of the grid is about 0.25 volt each side of free grid potential. At the tungsten string tension employed, a change in grid potential of 0.05 volt

results in a string shadow movement of about 1 cm. (0.2 meter per volt). The current flowing through the string (0.05 × 1500 = 75 microamperes) is about 400 times that necessary to produce a similar movement of a quartz string at conven-

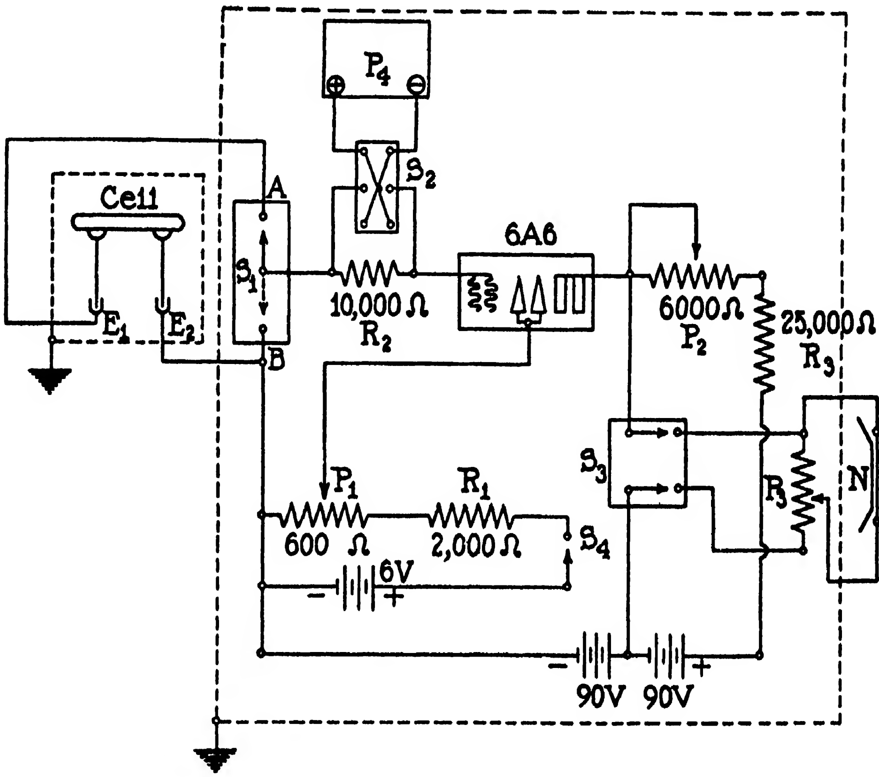

FIG. 4. Arrangement of amplifier.

S_1 S_2 S_3 = General Radio No. 339 B low capacity switches.
S_4 = toggle switch.
P_1 P_2 = General Radio No. 314 A potentiometers.
P_3 = 600 ohm No. 314 A General Radio potentiometer (for tungsten string).
= 20,000 ohm No. 314 A General Radio potentiometer (for quartz string).
P_4 = any available shielded potentiometer.
R_1 R_2 R_3 = International Resistance Co. 10 watt wire wound resistors.
E_1 E_2 = saturated calomel electrodes.
Cell = single cell of *Nitella*.

tional electrocardiogram tensions. Therefore the sensitivity with a quartz string instrument would be 0.050 ÷ 400 or 0.000125 volt per cm. (80 meters per volt).

In use, switch 1 is thrown to position *A* with no cell in the circuit, and P_2 adjusted until the galvanometer string is at zero. S_1 is then thrown to position *B*,

and P_1 is adjusted until the string is again at zero. This procedure is repeated once. With S_1 in position B, a calibrating potential is now applied across R_2, and P_3 is adjusted until the desired sensitivity is reached. Alternatively, the string tension may be adjusted. With the 3-string galvanometer, it is our custom to adjust the strings to approximately the same deflection with the same settings of the three potentiometers (P_3) and then make exact adjustments with the potentiometers. With a cell in the circuit, the calibrating potential is recorded. No difference will be found between the series and direct calibrations if the amplifier is in proper adjustment.

The apparatus is assembled in grounded iron boxes of the sort obtainable at radio supply stores, and all external wires are covered with grounded copper shielding. All controls have insulated shafts extending through the shielding.

All measurements were made from photographic records.

The plants are transported directly from the pond to the laboratory and immediately washed in tap water with as little mechanical manipulation as possible. They are then placed in Solution A[5] in enamel ware tubs covered with glass plates and kept in a cold room at 15° ±1°C.

To prepare a cell for experiments neighboring cells are cut away, leaving at each end a strip of dead cell wall about 10 mm. in length by which the cell can be picked up with bone-tipped forceps which do not actually touch the living cell.[6] The cells thus prepared are allowed to stand for several days in Solution A before being used.

The experiments described in this paper were made with flowing contacts, as shown in Fig. 5. A paraffin block P is shown in cross-section with a strip of filter paper F resting on it. The *Nitella* cell N rests on this and is covered with a thin layer of moist cotton C. Solution runs from the tube T over the cell and down to the cup B. The filter paper touches the tube T and the cup which in turn touches the waste beaker so that no drops are formed at any point. A continuous flow is maintained even during a change of solutions. For this purpose the old solution is allowed to run out until the funnel is nearly empty. The new solution is then poured in so that it follows the old solution without interruption.

Connections to the string galvanometer (through the calomel electrodes) are made as shown in Fig. 3. Care is taken to maintain a moist atmosphere around the exposed parts of the cell.

The pH of the solutions of inorganic salts between 6 and 9 has little effect on *Nitella* and no especial precautions on this score are needed.

The temperature varied between 20 and 21°C.

In making the record shown in Fig. 2, three places on the cell (C, D, and E, Fig. 3) were connected (through separate amplifiers and

[5] For the composition of this see Osterhout, W. J. V., and Hill, S. E., *J. Gen. Physiol.*, 1933–34, **17**, 87.

[6] *Cf.* Osterhout, W. J. V., *Biol. Rev.*, 1931, **6**, 369; *Ergebn. Physiol.*, 1933, **35**, 967.

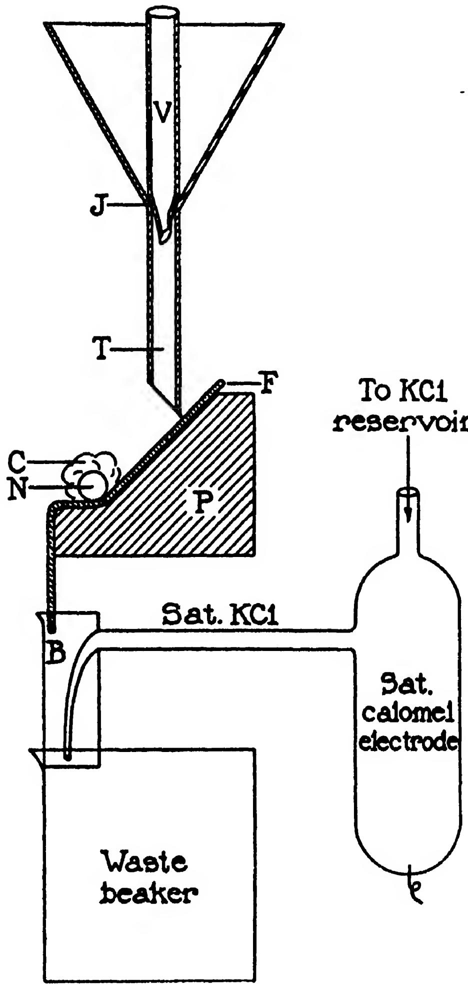

FIG. 5. Shows a cross-section of the arrangement of flowing contact. The solution is poured into the funnel. The rate of flow is determined by a groove in the ground joint *J*. Formation of slugs of water in the 3 mm. tube is prevented by the vent tube *V*. The solutions flow from the upright tube *T* to filter paper *F* which lies on a paraffin block *P*. On this rests a *Nitella* cell, *N*, covered with moist cotton, *C*; thus the solution flows completely around the cell. The filter paper (several layers) touches the tube *T* and enters the cup *B* filled with saturated KCl. Cup *B* overflows into a waste beaker which it touches so that formation of drops is precluded.

Connection to the calomel electrode is made by means of a tube filled with saturated KCl: this tube is fused with cup *B* and with the calomel electrode vessel. The bridge and cup are flushed out by a constant small flow of saturated KCl from a reservoir.

The P.D. of the liquid junction between the saturated KCl in cup *B* and the other solution at the top of cup *B* is in most cases negligible.

through the 3-string Einthoven galvanometer) to a spot F at the right end of the cell. The spot F was in contact with 0.01 M KCl which kept the P.D. constant, approximately at zero.[7] Any change in P.D. at F would cause simultaneous changes at C, D, and E. The absence of such changes was shown by the records[8] of C and E (omitted to save space). Hence we may be sure that all the alterations seen in Fig. 2 took place at D.

The record starts with 0.0001 M KCl at D which shows a positive[9] potential[10] of 125 mv. When the external concentration was raised to 0.000316 M the curve rose 7 mv. (indicating a loss of potential[11]).

When 0.001 M KCl was applied the curve jumped up 15 mv. This was soon followed by a gradual rise and an action current[12] after which the level of the curve remained considerably higher.

This raises some interesting questions which involve the structure of the protoplasm. We suppose that the protoplasm consists of an aqueous layer W (Fig. 3, p. 210) with an outer (X) and an inner (Y) non-aqueous layer. The outwardly directed (positive) P.D. appears to be due to an outward gradient[13] of K^+ across Y.

When the potential at D has been lowered by 22 mv. (by applying 0.000316 M KCl followed by 0.001 M) we may suppose that an adjoining region D_1, only a few microns from the edge of the drop of 0.001 M KCl covering D_1, discharges into D in the usual way.[13,14] This, of

[7] This was not measured on this cell but was determined for other cells of the same lot by leading off from a spot in contact with 0.01 M KCl to one in contact with 0.01 M KCl saturated with chloroform which latter reduces the P.D. to zero.

[8] C and E were in contact with pond water during the entire experiment.

[9] The potential is regarded as positive when positive current tends to flow from the sap across the protoplasm to the external solution.

[10] This value is reckoned from the zero given on the record which depends on the assumption that the P.D. at F is zero (*cf.* footnote 7).

[11] See earlier experiments, Osterhout, W. J. V., and Harris, E. S., *J. Gen. Physiol.*, 1928–29, **12**, 761; Osterhout, W. J. V., *J. Gen. Physiol.*, 1929–30, **13**, 715.

[12] It might be thought that this comes from mechanical stimulation but in that case the start of the action current would be abrupt and not gradual (*cf.* Osterhout, W. J. V., and Hill, S. E., *J. Gen. Physiol.*, 1930–31, **14**, 473). Mechanical stimulation is very improbable in view of the precautions taken to avoid it in changing solutions (see p. 212).

[13] Osterhout, W. J. V., *J. Gen. Physiol.*, 1934–35, **18**, 215.

[14] Osterhout, W. J. V., *Biol. Rev.*, 1931, **6**, 369.

course, is not recorded[15] at D. It may involve only a partial loss of P.D. at D_1 for we find that it is not propagated to C and E[16] (this is often the case with discharges involving incomplete loss of potential in *Nitella*).

We suppose that such a discharge involves an increase in permeability at D_1 accompanied by a movement of substances (organic and inorganic) from the sap into W. If these substances diffusing along W to D (only a few microns distant) cause an increase in the permeability[17] of Y at D we can understand why an action current occurs at D. The delay[18] after the application of 0.001 M KCl would be due to the time required for the diffusion of substances from D_1 to D in W.

The loss of potential due to the action current at D, amounting to 77 mv. at the spike, is presumably larger than at D_1. At any rate it is propagated and appears at C and E.

When an ascending series of concentrations of KCl is applied an action current is regularly encountered at 0.001 M to 0.005 M KCl.

We suppose therefore that the action current at D is brought about by the application of KCl which depresses the P.D. at D. But such a depression brought about by the application of NaCl seldom produces an action current.[19] This may be due to the fact that, as Blinks has shown,[20] KCl lowers the resistance of the protoplasm much more than NaCl does. The lowered resistance would facilitate the discharge of D_1 into D. It is possible that the presence of KCl in the external solution acts in other ways to facilitate the production of the action current.

[15] This is to be expected since there need be no change of P.D. at D. When a discharge occurs the change of P.D. takes place at the source and not at the sink. This is clearly shown when the sink is a dead spot.

[16] The records of C and E are omitted to save space.

[17] It may seem strange that Y which is in contact with sap at its inner surface should suffer an increase in permeability when sap reaches the outer surface. But this is less surprising when we remember that *Valonia* soon dies when placed in its own sap and that the process of death is accompanied by a great increase in permeability in both Y and X. *Cf.* Osterhout, W. J. V., *J. Gen. Physiol.*, 1924–25, **7**, 561.

[18] As would be expected, the duration of this delay is variable.

[19] When an action current occurs there is no extra loss.

[20] Blinks, L. R., *J. Gen. Physiol.*, 1929–30, **13**, 495.

When an action current is produced by KCl the subsequent level of the curve is higher than before. For convenience we shall refer to this as the "extra loss" of potential due to the action current. This extra loss is evident in the subsequent course of the curve even after the external KCl has been raised to 0.1 M and lowered again to 0.000316 M for we then find the curve at a higher level than when 0.000316 M KCl was first applied (Figs. 1 and 2).

In order to see whether the extra loss remains longer in evidence the external KCl was again raised to 0.1 M and lowered to 0.000316 M (stepwise as in Fig. 2). Above 0.001 M this curve practically duplicated that in Fig. 2 and the extra loss was in evidence throughout.

Is the extra loss due to changes in *X* or in *Y* or in both? Let us first discuss *X*. In previous experiments we have observed a permanent loss of potential after an action current. This has been explained as follows.[21] The spike of the action current is due to an increase in the permeability of *Y* which allows K^+ to move out of the sap (where its concentration is about 0.05 M) into *W*. This lessens the gradient[22] of K^+ across *Y* and hence lowers the outwardly directed (positive) potential. At the same time an organic substance, called for convenience[23] *R*, coming out of the sap makes *X* more sensitive to the action of K^+ and thus increases the inwardly directed (negative) potential due to the external KCl acting on *X*. We suppose that even if *Y* regains its original positive potential during recovery there remains the extra loss of potential due to the increased effect on *X* of the external KCl.

If the increased sensitivity of *X* to KCl is due to an organic substance *R* which comes out of the cell sap we might expect the extra loss of P.D. to persist as long as *R* remains in *X*. It would seem that there is one group of substances, which may be called R_P for convenience, which increases the sensitivity of *X* to KCl and another group, which may be called R_A, which facilitates the production of action currents. There is some unpublished evidence[24] that potassium

[21] Osterhout, W. J. V., and Hill, S. E., *J. Gen. Physiol.*, 1934–35, **18**, 681.

[22] The loss of P.D. might also be due to mechanical breaks in *Y*. *Cf.* Osterhout, W. J. V., and Harris, E. S., *J. Gen. Physiol.*, 1927–28, **11**, 673. Hill, S. E., *J. Gen. Physiol.*, 1934–35, **18**, 357. Osterhout, W. J. V., *Proc. Nat. Acad. Sc.*, 1938, **24**, 75.

[23] Hill, S. E., and Osterhout, W. J. V., *J. Gen. Physiol.*, 1934–35, **18**, 687.

[24] This will be discussed in another paper.

combines with a substance which we may call HZ to form KZ, which is identical with R_P. Hence we might expect that if K^+ is removed from the external solution the R_P in X would tend to lose its potassium and consequently to lose its efficiency. This seems to be the case. The extra sensitivity to KCl and consequently the extra loss of P.D. gradually disappears when the external solution of KCl is replaced by pond water, or by Solution A or by a solution of NaCl.[25] It may even disappear in a few minutes in 0.0001 M KCl.[26] In higher concentrations of KCl its disappearance is much slower. Theoretically we might expect it to disappear eventually even in the higher concentrations of KCl since it would tend to diffuse out into the external solution.

How does this extra loss of P.D. come about? Let us return to the equation given on p. 207 which may be written

$$\text{Change of P.D.} = 58\,\frac{U-V}{U+V}\log\frac{SC_1}{SC_2}$$

where C_1 and C_2 are the concentrations in the external solution and S is the partition coefficient (conc. in X ÷ conc. in the external solution). If S remains approximately constant[27] the slope of the curves in Fig. 1 will depend on the value of $(U - V) \div (U + V)$. Before the action current, when the concentration of KCl is raised from 0.000316 M to 0.001 M, we have (using concentrations for convenience in place of activities)

$$15 = 58\,\frac{U-V}{U+V}\log\frac{S\,0.001}{S\,0.000316}$$

$$\frac{15}{58\,(0.5)} = \frac{U-V}{U+V}$$

[25] In 0.01 M NaCl or in more dilute solutions it may disappear in less than a minute. The test is made by substituting NaCl for KCl and then replacing KCl of the same concentration as before to see whether the same P.D. is observed.

[26] This is most easily observed by treating the cell as in Fig. 2 and when the concentration has been lowered from 0.1 M to 0.0001 M KCl leaving it until the excess loss of potential gradually disappears, as shown by the gradual downward drift of the curve.

[27] This is probably true under normal conditions in the absence of action currents.

whence

$$(U - V) \div (U + V) = 0.52$$

When the next change of concentration is made we have

$$28 = 58 \frac{U - V}{U + V} \log \frac{0.00316}{0.001}$$

$$\frac{28}{58\,(0.5)} = \frac{U - V}{U + V}$$

whence $(U - V) \div (U + V) = 1$ (this can happen only when U is extremely large as compared to V).

Evidently therefore we cannot expect the change of P.D. to exceed[28] 28 mv. no matter how much the value of U is increased by the action of R coming out of the sap for it cannot raise the value of $U \div V$ above unity.

Apparently the action current causes R to come out of the sap and this raises the value of $(U - V) \div (U + V)$, so that the change in P.D. is 28 mv. instead of 15 mv. In other words the action of R adds $28 - 15 = 13$ mv. to the change of P.D. and this 13 mv. appears as part of the extra loss of P.D. But as the total extra loss is 60 mv. we still have $60 - 13 = 47$ mv. to account for. This extra 47 mv. must be due to the action of R in raising S to S'. The amount of this rise can be calculated as follows. We may write

$$\text{Change of P.D. due to change of } S \text{ to } S' = 58 \log \frac{S'}{S}$$

When this change is 47 mv. we have

$$47 = 58 \log \frac{S'}{S}$$

whence $S' \div S = 6.5$.

On this basis it would appear that we can distinguish between changes in partition coefficients and changes in mobility. It may be noted that this is not possible with the equations ordinarily used for phase boundary potentials.

[28] The value $0.001 \div 0.000316$ in the equation will be lessened when activities are employed.

Great changes in partition coefficients may be caused by the addition of organic substances as has been repeatedly shown in unpublished experiments on models in this laboratory.[29]

After the action current there is a considerable increase in the potassium effect; *i.e.*, the loss of P.D. produced by substituting a given concentration of KCl for the same concentration of NaCl. Before the action current this amounts to from 15 to 25 mv. After the action current it is 45 to 65 mv. This was also observed in earlier experiments.

We suppose that this indicates a greater increase in the partition coefficient[30] of KCl than in that of NaCl after the action current for the concentration effect of NaCl showed little or no change which indicates that the mobility[31] of Na^+ remained approximately constant. Hence the increase in the potassium effect must be due to changes in partition coefficient rather than in mobility. (For descending series see p. 221.)

It may be added that the experiment shown in Fig. 2 has been varied by placing the cell at first in 0.000316 M NaCl and then transferring to 0.000316 M KCl. In a typical experiment the curve jumped up 35 mv. when the KCl was applied. This was followed by a slow rise and an action current after which the level of the curve was 25 mv. higher than just before the action current. Thus the behavior of the curve before and after the action current resembled that in Fig. 2.

[29] The expression "partition coefficient" as here used should be interpreted in a very liberal sense to include such cases as the following. When 0.2 M $Ba(OH)_2$ in water is shaken with isoamyl alcohol the partition coefficient (Ba^{++} in amyl alcohol ÷ Ba^{++} in water) is less than 0.0005, but when the amyl alcohol contains 0.1 M oleic acid the partition coefficient rises to 0.029 because barium oleate is formed. When the aqueous solution of $Ba(OH)_2$ is 0.0015 M the partition coefficient rises to 3.46: the corresponding figure for $Ca(OH)_2$ is about 16.8. Temperature about 22°C.

[30] In earlier experiments the increase in the potassium effect involved an increase of the mobility of K^+, as shown by the increase in the slope of the curve of concentration potential (when plotted as in Fig. 1). *Cf.* Osterhout, W. J. V., *J. Gen. Physiol.*, 1934-35, **18**, 987.

[31] In these cells (Lot B) the mobility of Na^+ (as shown by the concentration effect) was much higher than in the cells studied earlier (Lot A).

This discussion indicates that we may account for the excess loss of potential by changes in X. But it is probable that changes occur in Y and it would seem natural to look to these to explain the fact that after the action current in Fig. 2 no subsequent action currents are produced[32] by further increases[33] in the concentration of KCl. This may also be due, in part at least, to the fact that on standing the diffusion boundary between D and D_1 becomes more diffuse and this makes more difficult a discharge from D_1 into D (this has been discussed in a previous paper[34]).

It is also possible that changes in Y might account, in part at least, for the excess loss of potential, *e.g.* by changing partition coefficients or mobilities or by a mechanical alteration (*e.g.* producing a "leaky" condition).[21]

All of these suggestions are put forward merely as working hypotheses which may serve to bring the facts under a common viewpoint. Future investigation must decide their actual value.

It seems desirable before leaving this subject to consider briefly the sources of these potentials. They are, of course, thermodynamic[35] as distinguished from zeta potentials. In previous studies the electrical behavior of the cell could be predicted by using the equations for diffusion potential[36] rather than those for phase boundary potential.[37] In consequence they have been regarded for convenience as diffusion potentials.

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[32] In a few cases a second action current occurred at the next increase in the concentration of KCl. Perhaps in these cases the changes produced by the first action current were less complete.

[33] Even when all the steps shown in Fig. 2 were immediately repeated on this cell no action current occurred.

[34] Osterhout, W. J. V., and Hill, S. E., *J. Gen. Physiol.*, 1929–30, **13**, 547.

[35] This is shown by the fact that they can be measured by means of a galvanometer. According to L. R. Blinks (personal communication) *Halicystis Osterhoutii*, Blinks and Blinks, can produce continuously for several days a current about 2.5 microamperes per cm.^{2} of cell surface. For technique see Blinks, L. R., *J. Gen. Physiol.*, 1935–36, **19**, 875.

[36] Osterhout, W. J. V., *J. Gen. Physiol.*, 1929–30, **13**, 715. Damon, E. B., *J. Gen. Physiol.*, 1932–33, **16**, 375.

[37] Donnan potentials need not be considered since nothing resembling a Donnan equilibrium exists and oxidation-reduction potentials are ruled out because no metallic electrodes were in contact with the cell (*cf.* Osterhout, W. J. V., and Hill, S. E., Cold Spring Harbor symposia on quantitative biology, Cold Spring Harbor, Long Island Biological Association, 1936, **4**, 43).

In the cells previously studied the rôle of K^+ was so predominant that other ions were neglected. For convenience we shall continue to do this but it should be understood that Na^+ plays a more important rôle in the present cells (Lot B) than in the earlier ones (Lot A). We know that potassium enters and reaches a much higher concentration in the sap than in the pond water.

One way in which P.D. might be brought about is seen in artificial cells[38] where the protoplasm is represented by guaiacol. When a dilute solution of potassium is placed outside the artificial cell potassium enters until its concentration in the artificial sap inside the cell is much higher than outside. During this process the potential due to the potassium compounds undergoes a change of sign. At first it is negative (inwardly directed) but as K^+ accumulates in the artificial sap the sign of the potential becomes positive (outwardly directed) as in *Nitella*.

We suppose that the positive potential in *Nitella* is due chiefly to compounds of potassium and sodium which reach a higher concentration in the sap than in the external solution. This appears to be chiefly due to a concentration gradient across Y, *i.e.* to a gradient from the sap, which contains about 0.05 M KCl and 0.05 M NaCl, across Y to W, which appears to contain very little of either.

We may suppose that when the cell is in pond water there is not much potential across X but when sufficient KCl or NaCl is added to the external solution an inwardly directed potential appears at X. We may make the usual assumption that the outermost portion of X comes almost instantaneously into equilibrium with the external solution and that the innermost portion of X likewise comes into equilibrium with W. During such experiments as are shown in Fig. 2 the penetration of KCl at one spot on the cell may not have much effect on W which is constantly stirred by protoplasmic movement.[39]

If the concentration of K^+ in W remains constant and the external concentration of KCl is raised from 0.001 M to 0.1 M it should theoretically make no difference as far as P.D. is concerned whether we do this suddenly or stepwise (as in Fig. 2). In other words it makes no difference theoretically whether the diffusion boundary in W is sharp or diffuse.[40] Practically, however, we do find differences in experiments on diffusion in aqueous solutions when more than one electrolyte is involved but they are irregular and not predictable.

When the external concentration is suddenly raised from 0.001 to 0.01 M we suppose that a sharp diffusion boundary is formed in X as discussed in a previous paper.[1]

Let us now consider briefly the effect of starting with 0.1 M KCl and proceeding stepwise in a descending series. The application of 0.1 M

[38] Osterhout, W. J. V., *J. Gen. Physiol.*, 1932–33, **16**, 157.

[39] W extends the whole length of the cell (usually 10 cm. or more) and the spot D where KCl is applied is only a centimeter in width. The thickness of W is undoubtedly much greater than that of X.

[40] MacInnes, D. A., and Yeh, Y. L., *J. Am. Chem. Soc.*, 1921, **43**, 2563. Brown, A. S., and MacInnes, D. A., *J. Am. Chem. Soc.*, 1935, **57**, 1356.

KCl causes an action current as would be expected from what is said earlier (p. 215) but there is no delay since the curve jumps up at once to beyond zero owing to the action of the external KCl on X plus the effect of the loss of P.D. at Y which is quickly superimposed.[41] As in the ascending series there is a partial recovery after which the extra loss persists. To avoid superimposition the curve in Fig. 1 has been made to pass through the origin and hence does not show the extra loss.

In this case we find that the potassium effect is greater in the concentrated than in the dilute solutions. This may be due, in part at least, to contamination of the dilute solutions.

The descending series of concentrations in Fig. 1 (lowest curve showing the average of five cells) gives a smooth curve. Since the slope of this curve approaches the theoretical limit[42] it is evident that the high value (85.45) for $U_K \div V_{Cl}$ given in a former paper is justified.

These results clearly show the importance of using methods of measurement which allow us to detect action currents. The information afforded by continuous records is often indispensable.

SUMMARY

Cells of *Nitella* have been studied which behave differently from those described in earlier papers. They show unexpectedly large changes in P.D. with certain concentrations of KCl. This is due to the production of action currents (these are recorded at the spot where KCl is applied).

A method is given for the separate evaluation of changes of P.D. due to partition coefficients and those due to mobilities.

A new amplifier and an improved flowing contact are described.

[41] This effect on Y appears more quickly than in the action current of the ascending series. This might be expected since 0.1 M KCl would lower the resistance of the protoplasm more than would lower concentrations.

[42] The theoretical slope becomes a little less when activities are taken into account. *Cf.* footnote 1.

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ISOLATION, CRYSTALLIZATION, AND PROPERTIES OF SWINE PEPSINOGEN

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In 1882 Langley (1) noticed that a slightly alkaline extract of gastric mucosa contained a material which was not pepsin but which changed into pepsin upon acidification of the extract. This alkali-stable material capable of being converted into pepsin is known as pepsinogen. Holter and Northrop (2) partially purified pepsinogen by fractional salt precipitation of an extract of swine stomach mucosae.

Method of Isolation

The total pepsinogen in an average swine stomach mucosa is about a gram of which at least 90 per cent is located in that third of the mucosa known as the fundus. The minced fundus mucosae were extracted with a bicarbonate-ammonium sulfate solution followed by precipitation of the soluble pepsinogen at a higher concentration of ammonium sulfate. By this one extraction and precipitation an 80 per cent yield of the total pepsinogen was obtained. The product was 50 times as pure as the starting material and half as pure as the final crystalline product, based on the protein nitrogen analyses. The material at this stage was nearly the same as the best preparations of Holter and Northrop (2). Further purification of pepsinogen by fractional salt precipitation was unsuccessful. The preparation still contained considerable quantities of carbohydrate as well as protein impurities which were removed by treatment with a copper hydroxide reagent.

When a washed copper hydroxide suspension at pH 6.0 was mixed with a solution of the pepsinogen described just above, practically all of the protein and carbohydrate materials were bound to the copper precipitate. Upon extracting this precipitate with pH 6.8

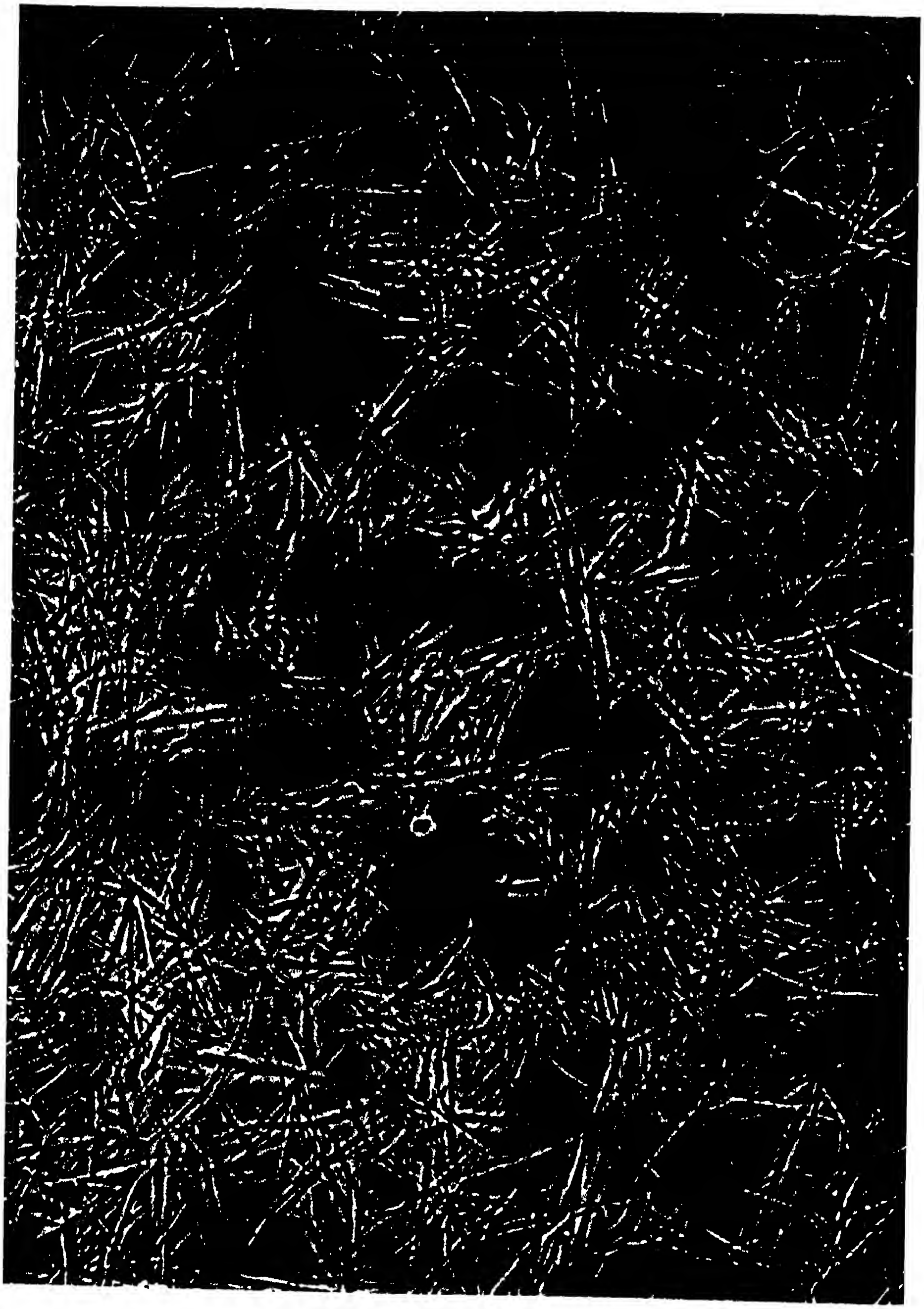

FIG. 1. Swine pepsinogen crystals.

phosphate buffer, 70 per cent of the pepsinogen went into the solution along with only a few per cent of the impurities. By repeating

this treatment with copper hydroxide a pepsinogen preparation was obtained which contained negligible quantities of carbohydrate and less than 5 per cent protein impurities. In fact no detectable improvement in the quality of the pepsinogen has been obtained with any subsequent treatment.

Crystallization of the pepsinogen after two treatments with the copper reagent was carried out by dissolving a 0.7 saturated ammonium sulfate filter cake in 9 volumes of 0.4 saturated ammonium sulfate-M/10 pH 6.3 phosphate buffer and stirring the solution slowly at 10°C. In 6–24 hours the beaker was a swirling mass of very thin long needles of crystalline pepsinogen (Fig. 1).

Tests of Purity

The first evidence of the purity of pepsinogen is a solubility experiment based on the phase rule which states that for a given solvent the solubility of a pure substance is independent of the quantity of solid phase; *i.e.*, the amount dissolved will not vary with the amount of solid. The solubility of pepsinogen in 0.55 saturated ammonium sulfate-M/10 6.8 phosphate buffer was found to be constant over a 20-fold variation in the quantity of solid pepsinogen. The quantity of pepsinogen dissolved was determined in three different ways, namely, estimation of the dissolved protein nitrogen and the amount of pepsin produced upon acidification as estimated by the hemoglobin and rennet methods.

The remaining evidence concerning the purity of pepsinogen is the negative results of numerous experiments designed to separate the hypothetically pure pepsinogen from any impurity. Since the fundamental and characteristic property of pepsinogen is its quantitative conversion into pepsin and since the activity of pepsin can be determined with a precision of nearly 5 per cent, it was this property of pepsinogen (designated as "potential activity") that was examined during the following experiments. The fractionation experiments consist of changing either a small percentage or a large percentage of the pepsinogen by some procedure and then determining the potential activity per milligram of protein nitrogen in either the small percentage that was altered or in the small percentage left unchanged. If a protein impurity is present in the preparation before fractionation then it would be expected that some one of the fractionation

procedures would at least partially separate it from the pure pepsinogen and in doing so would raise the potential activity per milligram of protein nitrogen in the other fraction. The procedure used in the first experiment was fractional recrystallization in which both the crystals and the mother liquor were analyzed. It was to be expected that any impurity would tend to come out with the crystals or remain in solution in the mother liquor in some quantity other than just in proportion to the quantity of pepsinogen. Likewise, experiments were performed in which fractionation was carried out after partial heat and alkali denaturation and after partial reversal of completely heat and alkali denatured pepsinogen. In these experiments it was expected that an impurity would be separated into one fraction and would thus raise the potential activity per milligram of protein nitrogen of the other. However, in no case was there any consistent or definite evidence of a fraction having higher potential activity per milligram of protein nitrogen. It is concluded, therefore, that the pepsinogen as isolated, having withstood the available tests for protein purity, is a pure protein to the extent that the methods are precise, about 5 per cent.

Properties of Pepsinogen

Table I contains a summary of the properties of pepsinogen and, for comparative purposes, the corresponding properties of pepsin have been included. In general it may be stated that those properties which depend for the most part on the whole molecule are much the same in the two proteins, whereas those properties which are more probably a function of particular groups or structural parts of the protein are in a number of instances different. Thus, the elementary analyses, molecular weights, absorption spectra, and optical rotation are qualitatively the same in pepsin and pepsinogen whereas the enzymatic activity, pH stability range, isoelectric point, amino nitrogen, titration curves, reversibility of heat and alkaline denaturation, and antigenicity (3) are quite different in the two proteins.

Denaturation, one of the most characteristic properties of proteins is so different in pepsin and pepsinogen as to be worthy of particular notice. Alkali denaturation of pepsin takes place at appreciable rates in solutions more alkaline than pH 6.0. This has recently been

TABLE I

Comparison of Properties of Swine Pepsin and Pepsinogen

Property	Crystalline form	Catalytic activity	Specific activity or specific potential activity $[P.U.]^{Hb}_{mg.\,P.N.}$	$[\alpha]^{23°C.}_{D}$ pH 5.6 per gram of dry protein	I.E.P.
Pepsin	Hexagonal bipyramids	+	0.26	−72°	pH 2.7
Pepsinogen	Needles	−	0.22	−62°	pH 3.8

Property	Molecular weight (osmotic pressure)	NH_3-N per cent of T.N.	Reversibility of denaturation	Hydration or molecular shape
Pepsin	38,000 ± 3,000	1.4	1.0 per cent	Hydrated or non-spherical
Pepsinogen	42,000 ± 3,000	4.	80–100 per cent	Non-hydrated and spherical

Property	pH-stability	pK of alkaline inactivation at 25°C.	Tyrosine-tryptophane content		Absorption spectrum	Elementary analyses; per cent of dry protein						
			at pH 11.	at pH 8.0		C	H	N Dumas	N Kjeldahl	S	P	Cl
Pepsin	pH 2.0–6.0	7.0	9.3	5.5	Same	51.7	6.8	14.7	14.0	0.45	0.09	0.00
Pepsinogen	pH 6.0–9.0	9.4	8.6	3.2	Same	52.8	6.9	14.2	13.9	0.4	0.08	0.00

studied in considerable detail by Steinhardt (4). The reaction follows a simple unimolecular course with an increase in rate as the pH increases and in concentrated solutions the denaturation is not reversible. Although a detailed study has not been made heat denaturation is not readily reversible and Northrop (5) has shown that it also followed a unimolecular course. Pepsinogen, on the other hand, although denatured by heat or in solutions more alkaline than pH 8.5, is denatured in a completely reversible manner. After complete denaturation, as demonstrated by its insolubility in salt solutions in which the native form is soluble and by the fact that the protein is not converted into pepsin when mixed with acid, the denaturation may be completely reversed and the pepsinogen shown to be native by the same criteria as were used to test for denaturation. Denaturation of pepsinogen is similar to the denaturation of trypsin by heat (6) or of hemoglobin by salicylate (7). That the denaturation of pepsinogen is a true equilibrium reaction was established by the fact that at any temperature or pH in heat or alkali denaturation the quantity denatured is the same whether that temperature or pH be approached from the higher or lower value, *i.e.* the quantity of native or denatured protein is the same when the conditions are approached from the completely native or completely denatured side of the equilibrium. Upon long exposure of pepsinogen to the denaturing agents the reversibly denatured material is slowly changed into an irreversible form. The rate of this change is increased by an increase in temperature or pH and, in the case of the change caused by heat, the reaction follows the course of a unimolecular reaction.

It may be seen from the titration curves of pepsin and pepsinogen that the total number of groups titrating between pH 6.0 and pH 12.0 is nearly the same in the two proteins but the pH at which the groups titrate is different; *i.e.*, the slope at any pH is not the same for the two proteins. However, the pH at which the groups titrate coincides with the pH at which the corresponding protein is rapidly denatured.

When solutions of pepsinogen are made more acid than pH 6.0 the protein is converted into pepsin. This conversion of pepsinogen into pepsin has been examined from two angles, the kinetic and chemical. In the kinetic study it was found that between pH 4.5–

5.0 the course of the conversion is accurately described by a simple autocatalytic equation. That the reaction is autocatalytic means that a product of the reaction, in this case pepsin, catalyzes the reaction and therefore produces more of itself. The reaction is at least partially autocatalytic at pH 0.0 as shown by an increase in the rate of conversion upon addition of pepsin. However, in solutions more acid than pH 4.0 the reaction appears to deviate from the simple autocatalytic. The rate of conversion is greatly increased as the pH decreases with a maximum near pH 1.0. The rate decreases beyond this maximum as the pH decreases and this decrease in activity does not appear to be an acid denaturing effect upon either pepsin or pepsinogen. The presence of salts in the medium has an accelerating effect upon the conversion. The concentration and charge or valency of the ions of the salt are variables which affect the rate. Kunitz and Northrop (8) have noticed a somewhat similar effect of salt on the autocatalytic conversion of trypsinogen into trypsin.

A chemical study of the conversion has shown that as pepsin is formed from pepsinogen there is a concomitant cleavage of the pepsinogen molecule. 15–20 per cent of the pepsinogen nitrogen appears in the solution as non-protein nitrogen; *i.e.*, nitrogen not precipitated by hot 2.5 per cent trichloracetic acid. It was demonstrated that this appearance of non-protein nitrogen in the solution was not the complete digestion of part of the pepsinogen by pepsin by the fact that addition of three times as much pepsin as pepsinogen resulted in the production of the same quantity of non-protein nitrogen upon activation as did the control where activation was spontaneous. It could be supposed that in spite of the tests for purity which showed the pepsinogen preparations to contain little or no protein impurity that there was 15 per cent of a foreign protein which consistently resisted fractionation. This was made less likely by some experiments which showed that under widely different conditions of activation the change in non-protein nitrogen varies linearly with the increase in activity and that under these same varied conditions the increase in non-protein nitrogen and activity stop at the same time.

Part, if not all, of this 15–20 per cent non-protein nitrogen is capable of acting as a dissociable inhibitor with pepsin. Its effect is most pronounced at pH 5.8 where the rennet activity of pepsin is

measured. Dissociation of the inhibitor pepsin complex takes place in acid so that there is no complicating effect on the hemoglobin activity measurement of activation mixtures. The amino nitrogen of an activation mixture is larger than the original pepsinogen by about nine amino groups per molecule of pepsinogen. It is to be expected that the conversion of pepsinogen into pepsin would result in an increase in amino nitrogen since pepsin is catalyzing the reaction and no linkage other than the peptide linkage is known to be split by pepsin.

It appears then, that upon acidification of a solution of pepsinogen an enzymatically inactive, reversibly denaturable protein with an isoelectric point of pH 3.8 is split at probably not more than nine peptide linkages to produce pepsin, an active, irreversibly denaturable protein isoelectric at pH 2.7, and a non-protein nitrogenous fragment which contains 15–20 per cent of the original pepsinogen nitrogen.

EXPERIMENTAL RESULTS

Preparation of Crystalline Pepsinogen

A detailed account of a typical preparation is presented in Table II. The text of this section includes a description of the first step in the preparation and a discussion of some of the procedures and materials used in the remainder of the preparation exemplified in Table II.

The mucosae used throughout this work have been prepared from stomachs of freshly killed swine. The stomach is removed immediately after death, turned inside out, and the contents washed away with cold water. No ill effects will be noticed if the stomachs remain after washing for as much as 5 hours at room temperature but a longer time is to be avoided.

The fundus part is the only desirable portion of the stomach and is accordingly cut out from the remainder with scissors. The fundus is pink or darker in color than the surrounding tissue and may easily be recognized when the stomach is inside out.

The layer of muscle tissue must be separated from the mucosa of the fundus. This is most expediently carried out by first carefully cutting the connecting tissue to allow one to grip the layers separately and then pulling in opposite directions. The fundus mucosa is then placed upon a flat surface and the clinging slime or mucous scraped off with the aid of a microscope slide or any piece of glass having a straight edge. This yellow mucous, if not removed, greatly retards the subsequent purification procedures. Analyses of swine gastric mucous have recently been made (9). Removal of the slime is followed by a rinse in cold water. The

mucosae are now ready for use and may be either minced and extracted immediately or stored in a freezing ice box. Fundus mucosae which had been stored at −13°C. for as long as 6 months exhibited no detectable difference from the freshly prepared ones with regard to either the handling or to the pepsinogen content.

A description of the preparation of the copper hydroxide suspension is to be found in the section of this paper devoted to Experimental methods. The pH of the copper hydroxide suspension is a factor of considerable importance. It must be pH 6.0 ± 0.2 as indicated by brom thymol blue and methyl red. Sulfuric acid and sodium hydroxide may be used to adjust the pH of the suspension to pH 6.0. If the pH is too acid (pH 5.5 or less) activation of pepsinogen takes place and if too alkaline (over pH 6.3) the reaction between the copper suspension and the pepsinogen solution does not give the desired results. Titration of the pepsinogen solution to pH 6.0 ± 0.2 is also necessary for the same reasons. This titration is best carried out with 4 M pH 5.0 acetate buffer so that at no time is any portion of the solution below pH 5.0.

Pepsinogen of 1.0–1.5 mg. protein nitrogen per ml. concentration is completely removed from solution at pH 6.0 when mixed with an equal volume of washed copper hydroxide suspension of about molar concentration. Most of the soluble carbohydrate goes with the pepsinogen and the copper hydroxide.

The phosphate-copper-pepsinogen suspension usually has an acidity of pH 6.5 to brom thymol blue. After filtration of this suspension and washing the residue as directed, most of the pepsinogen is found in the filtrate along with only small amounts of impurities.

Crystallization

After the second or last copper treatment followed by filtration with 5 per cent Filter Cel, the pepsinogen is precipitated in the following manner: the solution is titrated to pH 6.0 with 4 M pH 5.0 acetate and brought to about 0.4 saturation by the addition of 242 gm. solid ammonium sulfate per liter of solution. Now with slow but constant stirring a volume of saturated ammonium sulfate equal to the volume of the 0.4 saturated ammonium sulfate-protein solution is added through a capillary tube dipping below the surface of the liquid. Instead of filtering, the suspension is now stored for 1 or 2 days at 5–10°C. After this time the suspension is filtered with suction on a C.S. and S. No. 575 hardened filter paper without the aid of any Celite and the residue is then stirred in 9 volumes of 0.40–0.42 saturated ammonium sulfate in M/10 pH 6.25 phosphate buffer at 10°C. The precipitate usually dissolves but it is very sensitive to the ammonium sulfate concentration. In the event it does not dissolve M/10 pH 6.25 phosphate should be added dropwise until the precipitate goes into solution. Although pH 6.25 phosphate is used the pH of the crystallizing mixture is about pH 5.0 as determined by both the quinhydrone and glass electrodes. This change is due to the effect of the high concentration of salt. If this solution is now stirred slowly but constantly the pepsinogen

TABLE II

Preparation of Crystalline Pepsinogen from Swine Gastric Mucosae

Procedure, details of preparation, etc.	No.	Time of handling	Vol.	Protein nitrogen	Potential $[P.U.]^{Hb}$*				Carbohydrate (as glucose)	
		hrs.	*ml.*	*mg./ml.*	*1/ml.*	*1/mg. P.N.*	*total*	*per cent of original*	*mg./ml.*	*1/[P.U.]Hb*
3400 gm. of twice minced, prepared, frozen swine gastric fundus mucosae mixed with 4 times its weight of 0.45 sat. ammonium sulfate in M/10 sodium bicarbonate; stirred 1 hr.	1	1.5	16000	0.67	0.042	0.07	670	100	0.68	14.0
To No. 1 was added 100 gm. fine "Filter Cel" and 50 gm. coarse (Hyflow) Cel per liter of 0.45 sat. ammonium sulfate solution used. Stirred 15 min. and filtered on 30 cm. Buchner funnel with Whatman No. 3 paper covered with a thin cake of coarse Cel. Filtrate clear. When cake was nearly dry it was washed on funnel twice with 300–500 ml. of 0.45 sat. ammonium sulfate in M/10 sodium bicarbonate, residue discarded; filtrate and washings	2	2.0	13000	0.37	0.048	0.13	630	94	0.24	5.0
No. 2 was brought to 0.68 sat. by addition of 180 gm. solid ammonium sulfate, per liter. Solid ammonium sulfate allowed to stand 10 min. in solution before stirring and then stirred slowly. The suspension allowed to settle overnight or filtered immediately after addition of 50–100 gm. coarse Cel. Discard filtrate	3	0.5	14000		0.0022		30	5	0.16	73.0
Above residue dissolved in water and Cel filtered off. Cel was washed on the funnel until a sample of the washing showed no protein present. Filtrate and washings were combined	4	0.3–0.5	3500	1.3	0.15	0.12	530	79	0.3	2.0

No. 4 was titrated to pH 6.0 ± 0.2 (yellow to methyl red and to brom thymol blue) with 4 M pH 4.65 acetate buffer and mixed with equal vol. M/1 copper hydroxide suspension previously titrated to pH 6.0. Mixture stirred a few minutes and filtered on large Buchner funnels. Filtrate contained no protein as shown by heating 3 ml. of filtrate with 3 ml. of 10 per cent trichloracetic acid. If protein is found more copper suspension must be added and refiltered. Final filtrates were discarded. Filtrate	5	2–3	6500		0.003		20	3	0.053	17.0
Copper residue from No. 5 was stirred to a smooth paste with a vol. of M/10 pH 6.8 phosphate equal to that vol. occupied by the protein solution just before mixing with the copper suspension. This is also the vol. of copper suspension used. In this experiment it was 3500 ml. The residue was filtered and washed twice with 250 ml. M/10 pH 6.8 phosphate. Residue discarded and the filtrates and washings combined. Filtrates	6	1.5	5000	0.33	0.075	0.23	375	55	0.03	0.4
To No. 6 was added 5 per cent fine (Filter Cel) Cel, stirred and filtered, residue washed twice with 1 vol. M/10 pH 6.8 phosphate equal in ml. to the weight in gm. of fine Cel used. Residue discarded. Filtrate	7	0.25	5100	0.33	0.071	0.20	360	54	0.017	0.24

* These values represent the pepsin activity after complete activation of aliquots of pepsinogen. For details of measurements see the section on Experimental methods.

TABLE II—*Concluded*

Procedure, details of preparation, etc.	No.	Time of handling	Vol.	Protein nitrogen	Potential $[P.U.]_{Hb}$*				Carbohydrate (as glucose)	
		hrs.	*ml.*	*mg./ml.*	*1/ml.*	*1/mg. P.N.*	*total*	*per cent of original*	*mg./ml.*	$1/[P.U.]_{Hb}$
No. 7 brought to 0.7 sat. by addition of 474 gm. of solid ammonium sulfate per liter. 50–100 gm. coarse Cel added and suspension filtered. Filtrate discarded and residue stirred with water and separated from the Celite as described in No. 4. Solution should be diluted with water until the concentration of P.N/ml. is about 1 mg. Solution titrated to pH 6.0 as before and copper hydroxide treatment repeated as well as the treatment with 5 per cent fine Cel as described in Nos. 5, 6, and 7. Solution brought to 0.4 sat. by adding 242 gm. solid ammonium sulfate per liter. An equal vol. of sat. ammonium sulfate solution was added through a capillary tubing. Solution should be stirred slowly but constantly. Suspension allowed to stand 2 days at 10°C. and the protein was filtered *without Celite filter aids* on a Buchner with hardened filter paper. (A sample of residue was analyzed and figures were calculated for the entire quantity.)	8	6–8 and 8 standing				0.25	320	48		0.03
No. 8 as a 0.7 sat. ammonium sulfate filter cake was stirred with 9 vol. 0.40 sat. ammonium sulfate solution in M/10 pH 6.25 phosphate at 10°C. The solution was almost clear, containing dirt and shreds of filter paper and was therefore filtered. Solution on stirring slowly at 10°C. for a few hours became opalescent and a silkiness appeared which grew heavier. After stirring 2 days suspension filtered. Filtrate	9		460	2.1	0.43	0.21	200	30		
Crystal cake dissolved	10		126	3.5	0.74	0.21	94	14		

will crystallize out slowly in long, very thin, fiber-like needles. That crystallization has taken place is first apparent from the silkiness or swirl of the precipitate in the pepsinogen solution. If there is a precipitate but no swirling when stirred, microscopic examination will probably show either typical amorphous material or large spheroids. This condition is brought about by either too high a concentration of ammonium sulfate or incorrect hydrogen ion concentration. It indicates that the pepsinogen has come out too fast. If no precipitate appears in 12–24 hours either or both reasons may be responsible. The ammonium sulfate concentration may be too low or activation of some pepsinogen may have taken place resulting in a mixture of pepsin and pepsinogen from which pepsinogen would not crystallize.

Once crystallization takes place the system is allowed to remain unchanged for 24–36 hours. The crystals may then be filtered off or, if a larger yield is desired, the suspension is poured into an evaporating dish, stirred gently, and a stream of air passed over the surface. Other methods of forcing crystallization have not been successful.

Fig. 1 is a photograph of pepsinogen crystals. The crystals as they first come out are very thin or narrow and may be missed even by microscopic examination. The best identification of their being crystals is the swirl of a stirred suspension. At times the crystals are so small in diameter that they pass through the filter papers at first and then plug the paper so that filtration is very slow. Although Celite products help somewhat it still required 6–24 hours to filter a few hundred milliliters of suspension. Centrifugation has not been more successful.

Recrystallization

Filter cakes of crystalline pepsinogen differ so radically in their protein content, due to difficulties in filtration, that it is impracticable to outline a definite scheme for recrystallization. In general, the procedure is that used in the original crystallization except that precautions are taken to inactivate any pepsin which may have formed. Since activation is autocatalytic the amount of pepsin present must be minimal if crystallization of pepsinogen is to take place. This is most easily accomplished by making the pepsinogen alkaline just before commencing recrystallization. The simplest scheme is to dissolve the crystalline filter cake in the minimal amount of cold water, titrate it to pH 8.3 (just pink to 0.5 per cent phenolphthalein), warm to 40°C. for 5–10 minutes, cool to 10°C., and titrate to pH 6.2 (greenish yellow to brom thymol blue) with 4 M pH 4.65 acetate, and then add amounts of saturated ammonium sulfate and M/5 pH 6.25 phosphate to bring the final concentration of protein to 4–5 mg. per ml. and the ammonium sulfate concentration to 0.40–0.43 saturated. If the solution is not clear it should be filtered. Crystallization should proceed as in the original crystallization procedure. If it does not crystallize, precipitate the protein by slowly adding an equal volume of saturated ammonium sulfate and treat as in the scheme for the original crystallization.

Storage

Pepsinogen may be kept as a 0.7 saturated ammonium sulfate filter cake in a closed container at 5–10°C. The writer has also found that no loss of activity occurs if a dialyzed solution of pepsinogen is frozen in carbon dioxide snow ("Dry Ice") and then dried *in vacuo* below 0°C. Although it is too soon to speak from experience with this protein it is to be expected that as a dry powder it will keep better than as a filter cake.

Tests of Purity

Solubility Experiment.—The solubility is represented graphically in Fig. 2.

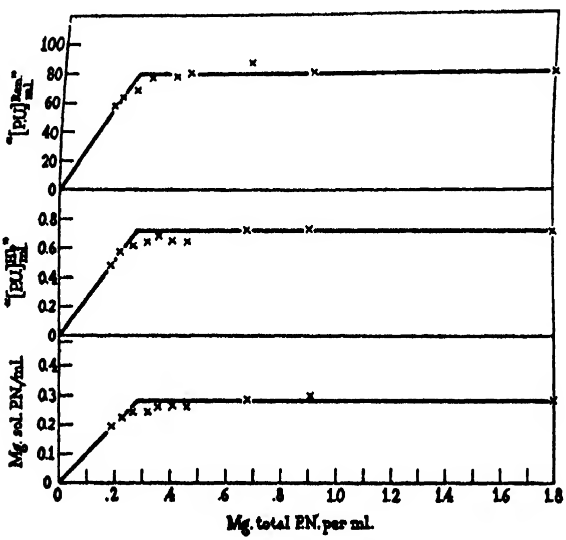

FIG. 2. Solubility of pepsinogen in 0.55 saturated ammonium sulfate—M/10 pH 6.8 phosphate at 21°C.

Experimental Procedure

The solubility experiments were carried out at 21°C. after first bringing to equilibrium the filter cake of twice crystallized pepsinogen and the solvent (M/50 pH 6.8 phosphate – 0.55 saturated ammonium sulfate). This was accomplished by stirring the cake with a succession of 100 ml. aliquots of the solvent until three consecutive filtrates were the same with respect to activity and protein nitrogen. Different amounts of the suspension were then added to a series of tubes and supplementary amounts of solvent were added to each tube to bring them to the same volume. 2.25 ml. of 0.0444 M phosphate buffer was then added to each tube which brought about solution of the precipitate. With rapid stirring 2.75 ml. of saturated ammonium sulfate was added. The suspensions were filtered and

aliquots of the filtrates activated and analyzed for activity by the hemoglobin and rennet methods. Other aliquots of the filtrates were analyzed for protein nitrogen after diluting to contain approximately 0.03 mg. protein nitrogen per ml. The analysis was carried out by adding 1.0 ml. of the diluted solution to 10.0 ml. of boiling 2.5 per cent trichloracetic acid and measuring the quantity of precipitate formed in a Duboscq type photoelectric colorimeter. A solution of copper sulfate was used as the standard in the colorimeter and a reference curve was obtained using known quantities of pepsinogen similarly treated.

TABLE III

Fractional Crystallization of Pepsinogen

Material analyzed	Protein calculated from P.N. analyses	$[\mathrm{P.U.}]^{\mathrm{Hb}}_{\mathrm{mg.\ P.N.}}$	$[\alpha]^{25°\mathrm{C.\ pH\ 6.3}}_{\mathrm{D\ per\ gm.\ protein}}$	Optical rotation* / $[\mathrm{P.U.}]^{\mathrm{Hb}}_{\mathrm{ml.}}$
	gm.			
Mother liquor of 1st crystallization.........	4	0.22	−67	2.1
Filter cake " " "	13	0.21	−58	2.0
Mother liquor of 2nd crystallization........	1	0.21		
Filter cake " " "	12	0.23	−66	2.0
Mother liquor of 3rd crystallization†	4	0.25	−71	2.0
Filter cake " " "	5	0.23	−67	2.0

* This figure is the ratio of the optical rotation of a solution (not specific optical rotation [α]) to the hemoglobin activity per milliliter of the same solution. This ratio does not depend upon the nitrogen measurement as do the other two values.

† Analyses of this mother liquor revealed that activation had taken place to a high degree. This is indicated also in the high $[\alpha]_{\mathrm{D}}$ and $[\mathrm{P.U.}]^{\mathrm{Hb}}_{\mathrm{mg.\ P.N.}}$

Fractionation Experiments.—Fractionation of pepsinogen was carried out by a number of procedures which are summarized as follows:

A.—Three fractional recrystallizations in which all fractions were analyzed for protein nitrogen, hemoglobin activity, and optical rotation. These results are to be found in Table III.

B.–Salt fractionation after partial heat denaturation at pH 7.0 and 55°C., 60°C., and 65°C.

C.—Salt fractionation after partial reversal at pH 7.0 and 55°C., 60°C., and 65°C. of completely (70°C.) denatured pepsinogen.

D.—Salt fractionation after partial denaturation at 25°C. and pH 9.1, 9.4, and 9.8.

E.—Salt fractionation after partial reversal at 25°C. and pH 9.5, 8.5, and 8.3 of completely (pH 10.5) denatured pepsinogen.

Fractions in B, C, D, and E, were analyzed for protein nitrogen and hemoglobin activity and the results are given in Table IV. These experiments are a part of those described in the sections on *Reversible heat denaturation* and *Reversible alkali denaturation.*

TABLE IV

Fractionation of Pepsinogen

	Conditions or procedures in fractionation		Original	Potential $[\text{P.U.}]_{\text{mg. P.N.}}$
			per cent	
B.	Soluble in M/1 magnesium sulfate at pH 7.0	Heat denaturation at 55°C.	87.0	0.22
		" " " 60°C.	46.0	0.20
		" " " 65°C.	8.0	0.21
C.		Reversal of heat (70°C.) denaturation at 65°C.	4.5	0.19
		" " " " " " 60°C.	40.0	0.20
		" " " " " " 55°C.	74.0	0.21
		" " " " " " 50°C.	93.0	0.22
D.	Soluble in M/1 magnesium sulfate at pH 5.0	Alkali denaturation at pH 9.1	78.0	0.22
		" " " pH 9.4	52.0	0.20
		" " " pH 9.8	13.0	0.22
E.		Reversal of pH 10.5 denaturation at pH 9.5	44.0	0.22
		" " " " " " pH 8.5	78.0	0.22
		" " " " " for 15 seconds at pH 8.3	24.0	0.23

Properties of Pepsinogen

Elementary Analyses

The analyses are given in Table V. The high ash content in all preparations was mostly sodium and magnesium which remained combined with the protein on the alkaline side of the isoelectric point, even on long dialysis.

The percentage sulfur is less than that reported by Northrop (5) but it is nearly the same for pepsin and pepsinogen.

Pepsin from Pepsinogen

It is of primary importance that the material isolated and called pepsinogen yield pepsin upon activation. Consequently crystalline pepsinogen was activated and the pepsin thus formed twice crystallized. This material was analyzed along with a sample of crystalline pepsin prepared in the manner described by Northrop (5) from commercial pepsin.

TABLE V

*Elementary Analyses**

Dialyzed and Dried in vacuo at 80°C.

Materials	C	H	N Dumas	N† Kjeldahl	S	P	Cl	Ash
	per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
2 × crystallized Cudahy pepsin	51.74	6.54	14.76	13.8	0.49	0.15	0.00	2.17
2 × crystallized Parke Davis pepsin	51.70	6.58	14.83	14.1	0.51	0.11 0.09‡	0.00	1.70
2 × crystallized pepsin from pepsinogen	51.61	6.86	14.63	13.9	0.42	0.09 0.09‡	0.00	1.78
Uncrystallized pepsinogen	52.80	6.88	15.92	15.1	0.37	0.09 0.084‡	0.00	1.72
Uncrystallized pepsinogen			14.40	13.8		0.084‡		
3 × crystallized pepsinogen			14.45	14.3				
3 × crystallized (Na) pepsinogen			14.00	14.00				
3 × crystallized No. 9 pepsinogen			13.59	13.4				

* Unless otherwise noted the analyses were micro-analyses and were made by Dr. A. Elek of Dr. P. A. Levene's laboratories.

† The Kjeldahl nitrogen estimations were carried out as previously described (11).

‡ Carried out by the writer using the method of Sørensen (10).

Most of the analyses and comparisons are to be found in the tables to follow under the specific properties. The crystalline form, optical rotation, and specific activities of the pepsin prepared from crystalline pepsinogen were similar to those properties of crystalline pepsins from commercial preparations. This is, of course, to be expected since the commercial pepsin is swine pepsin and came originally from swine pepsinogen.

Proteolytic Activity of Pepsinogen

It was of interest to see if pepsinogen had any proteolytic activity. Most pepsin activity measurements are carried out under conditions which would normally activate pepsinogen rapidly. However, two methods of estimation, the rennet and gelatin viscosity methods are carried out at pH 5.8 and 4.7 respectively. At these acidities the extent of activation of pepsinogen is not appreciable in the time required for measurement.

TABLE VI

Specific Activities

Materials	Methods*			
	Hemoglobin $[\text{P.U.}]^{\text{Hb}}_{\text{mg. P.N.}}$	Rennet† $[\text{P.U.}]^{\text{Ren.}}_{\text{mg. P.N.}}$	Gel. viscosity $[\text{P.U.}]^{\text{Gel. V.}}_{\text{mg. P.N.}}$	Edestin N P.N. $[\text{P.U.}]^{\text{Ed. sol.}}_{\text{mg. P.N.}}$
5 × crystallized Parke Davis pepsin	0.21	475	13	0.50
3 × crystallized Parke Davis pepsin 9–19–33	0.22	410	18	0.44
2 × crystallized Cudahy pepsin 12–7–33	0.30	435	33	0.57
3 × crystallized Cudahy pepsin 43–12	0.26	470	33	0.55
Crystalline pepsin from crystalline pepsinogen	0.26	360	12	0.48
3 × crystallized pepsinogen		<1	<1	
Specific potential activities‡ of pepsinogen preparations				
3 × crystallized pepsinogen	0.20	275	17	0.42
Average of 10 preparations of pepsinogen	0.22	250–300		
3 copper treatment but uncrystallized pepsinogen	0.21	270	17	0.46

* With the exception of the rennet method the methods were carried out as described by Northrop (12).

† Described in detail in the section devoted to Experimental methods.

‡ Explained in the text of this paper.

As seen from Table VI, pepsinogen is practically inactive proteolytically, as measured by its milk clotting activity and its action on gelatin.

Reversible Heat Denaturation

In the absence of salt, pepsinogen solutions may be heated to 100°C. without the formation of a visible precipitate. Upon cooling the

solution and analyzing for pepsinogen it is found that a large proportion of the pepsinogen is indistinguishable from the pepsinogen before heating. That heating to 70–100°C. actually denatures pepsinogen is indicated by the fact that a salt solution (M/1 magnesium sulfate) just sufficient to precipitate the pepsinogen above 70°C. has no precipitating effect on the pepsinogen at 50°C., nor upon the "reversed" pepsinogen. Also the soluble denatured protein is not converted into pepsin upon acidification. Two independent methods of estimating the extent of denaturation were based on these facts and were used in the experiments shown in Fig. 3.

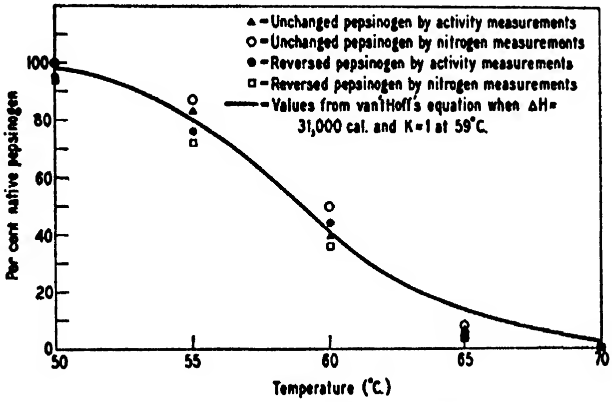

FIG. 3. Effect of temperature on the equilibrium between native and denatured pepsinogen at pH 7.0.

Experimental Procedure

Pepsinogen Solution.—1 mg. protein nitrogen per ml. of a dialyzed pepsinogen preparation at pH 7.0.

Procedure, I.—Approaching from the native or "activatable" side of the equilibrium; to each of two 30 ml. test tubes was added 5 ml. of the pepsinogen solution. These tubes were kept in a water bath at the desired temperature (50°C., 55°C., 60°C., 65°C., or 70°C.) for 10 minutes after which the solution in one tube was mixed with 5 ml. of 2 M magnesium sulfate at the same temperature, while the solution in the other tube was mixed with 5 ml. of N/20 hydrochloric acid also at the same temperature. The solution containing the magnesium sulfate was filtered and an analysis for nitrogen by the Kjeldahl method was performed on the clear filtrate. The solution to which had been added the N/20 hydrochloric acid was allowed to stand at 35°C. for 5 minutes after which it was diluted and activity measurements performed by the hemoglobin method.

II.—In approaching from the denatured or "unactivatable" side of the equilibrium two 5 ml. portions of the pepsinogen solution were heated to 70°C. for 5 minutes followed by cooling to the desired (50°C., 55°C., 60°C., 65°C.) temperature

for 5 minutes after which 1 aliquot was mixed with 5 ml. of 2 M magnesium sulfate while the other was mixed with N/20 hydrochloric acid. Both of these reagents were kept at the temperature to which the protein solution had been cooled. The solutions were then analyzed as described in I for the solutions which were heated to the temperature for 10 minutes.

At about 59°C. pH 7.0 and with 1.0 mg. protein nitrogen per ml. the denaturation equilibrium of pepsinogen is near the mid-point; *i.e.*, half the protein is denatured and half is native. The value of ΔH, the energy of the reaction, taken from van't Hoff's equation

$$\ln K = -\frac{\Delta H}{RT} + C$$

is 31,000 calories in the equilibrium shown in Fig. 3.

Secondary Heat Change

Heating to 100°C. gradually converts reversibly denatured pepsinogen into an irreversible form. This may be seen graphically in Fig. 4

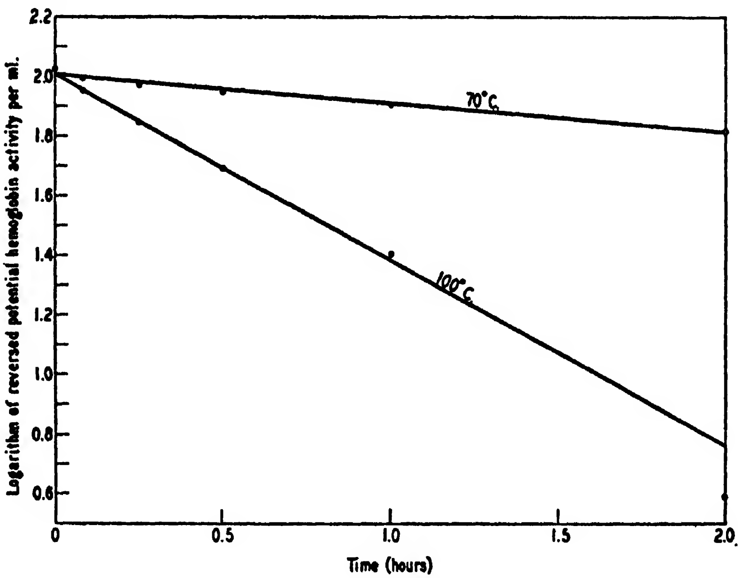

FIG. 4. Rate of change of reversibly denatured pepsinogen into an irreversible form at 70°C. and 100°C.

where the logarithm of the reversible potential hemoglobin activity is plotted against the time. It is apparent that whatever the nature of the change it follows a first order reaction.

Experimental Procedure

Two 15 ml. aliquots of a dialyzed pepsinogen solution at pH 7.0 containing 1.0 mg. protein nitrogen per ml. were heated at 70°C. and 100°C. At intervals of time 2 ml. samples were removed, cooled to 35°C. for 30 minutes, followed by addition of 2 ml. of N/20 hydrochloric acid. After 10 minutes allowed for activation the samples were diluted and the activity determined by the hemoglobin method.

Reversible Alkali Denaturation

Alkali denaturation of pepsinogen is a true equilibrium reaction as shown in Fig. 5, by the fact that at any pH between pH 8.5–10.5 nearly the same amount of native or denatured pepsinogen is obtained regardless of whether that pH is arrived at by raising the pH

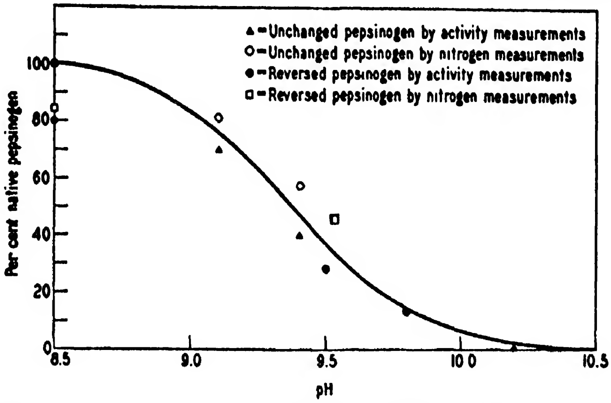

FIG. 5. Effect of pH on the equilibrium between native and denatured pepsinogen at 25°C.[1]

of a native activatable pepsinogen solution or by lowering the pH of a denatured unactivatable solution. The criteria of alkali denaturation, as in the case of reversible heat denaturation, is the insolubility in certain salt solutions in which native pepsinogen is

[1] If the logarithm of the ratio of native to denatured pepsinogen is plotted against pH, a straight line with a slope of 2 is obtained. This agrees with the assumption that the equilibrium between native and denatured pepsinogen is expressed by the following reaction—

$$\underset{\text{Pepsinogen}}{\text{Native}} + 2\,OH^- \rightleftharpoons \underset{\text{Pepsinogen }(OH^-)_2}{\text{Denatured}}$$

or

$$\log \frac{[\text{Pepsinogen}]}{[\text{Pepsinogen }(OH^-)_2]} = 2 \log [OH^-] + \log K$$

soluble and the fact that denatured pepsinogen does not change into pepsin upon acidification. It may be seen that pH 8.5 is the best pH to reverse the alkali denatured pepsinogen.

Experimental Procedure

Pepsinogen Solution.—10 mg. protein nitrogen per ml., pH 6.5 dialyzed salt free.

pH.—Determined by hydrogen electrode on mixtures of pepsinogen and the buffer. Electrode standardized in this region against Sørensen glycine and borate buffers.

Procedure, I.—Approaching from the native side of the equilibrium; two 0.5 ml. aliquots of the pepsinogen solution were added to two 4.5 ml. aliquots of M/10 borate buffer of various pH values. After standing 5 minutes at 25°C., 5 ml. of a 2 M magnesium sulfate in hydrochloric acid and 0.4 M pH 4.65 acetate was added to one aliquot and 5 ml. of N/5 hydrochloric acid was added to the other. The hydrochloric acid in the salt solution was of such a concentration as to just neutralize the borate buffer. The protein salt solution was filtered and the nitrogen determined in the clear filtrate. Activity estimations by the hemoglobin method were made on N/5 hydrochloric acid-protein solution.

II.—Approaching from the denatured side; 0.5 ml. aliquots were mixed with 4.5 ml. aliquots of M/10 pH 11.0 borate buffer, the final pH being 10.5, were allowed to stand 1 minute at 25°C. which was found to be long enough to completely denature pepsinogen. They were then mixed with 5 ml. of hydrochloric acid of just sufficient strength to change the pH to a desired value (pH 9.4 or 8.5) where the solutions were allowed to remain for 4 minutes. At this time, one aliquot of pepsinogen was mixed with 10 ml. 2 M magnesium sulfate in 0.4 M pH 4.65 acetate while the other was mixed with 10 ml. N/5 hydrochloric acid. These aliquots were then analyzed for nitrogen and activity as in *I*, the difference in dilution being considered.

The alkali denaturation of pepsin is almost an irreversible reaction when the protein concentration is comparable to that used in the pepsinogen experiments. A careful study by Steinhardt (4) brings out that the alkaline inactivation of pepsin is a first order reaction the rate of which is a function of the ionization of five titratable groups. He has suggested that the amino groups of cystine in pepsin may be involved. This seems unlikely to the writer who found that acetylation of the amino groups of pepsin had no detectable effect on the alkaline inactivation of the acetylated enzyme (13).

Secondary Alkali Change

Between pH 8.5 and 10.5 practically complete reversal of denaturation can be effected if the exposure to the alkali is for a short time.

As the time of standing increases the extent of the reversal decreases. Fig. 6 shows the results of an experiment in which a solution of pepsinogen stood at pH 10.5 and 25°C. for varying lengths of time after which reversal of denatured pepsinogen was allowed to take place for 30 minutes and the extent of reversal then determined. It may be seen from Fig. 6 that the denatured pepsinogen slowly changes into an irreversibly denatured form.

Experimental Procedure

0.5 ml. of a dialyzed solution of pepsinogen at pH 7.0 containing 10 mg. protein nitrogen per ml. was added to 4.5 ml. of M/10 pH 11.0 borate buffer bringing the pH to 10.5. At intervals of time 1 ml. of this solution was added to 1 ml. of 0.075 N hydrochloric acid which changed the solution to pH 8.5. This was allowed to

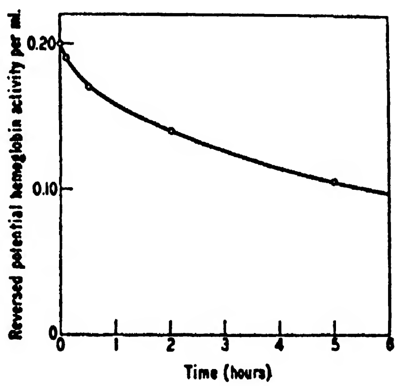

FIG. 6. Rate of change of reversibly denatured pepsinogen into an irreversible form at pH 10.5 and 25°C.

TABLE VII

Amino Nitrogen as Per Cent of Total Nitrogen

Material	Formol titration	Van Slyke gasometric
Pepsin Native	1.4	1.4
Denatured	1.4	1.4
Pepsinogen Native	4.4	3.5
Denatured	4.2	2.5

stand at 25°C. for 15 minutes, which is sufficient to completely reverse the alkaline denaturation. After this 2 ml. of M/10 hydrochloric acid was added and 10 minutes allowed for complete conversion of pepsinogen into pepsin after which it was diluted and the activity estimated by the hemoglobin method.

Amino Nitrogen

Table VII contains the amino nitrogen analysis of pepsinogen with pepsin as a control. Apparently the pepsinogen reacts with nitrous acid before it becomes activated since the value by the Van Slyke gasometric nitrous acid method (14) is not more than that by the formol titration, the latter method being carried out above pH 7.0. Pepsin contains less amino nitrogen than pepsinogen. This difference will be discussed in more detail later but it may be said here that part of the pepsinogen containing a relatively large amount of amino nitrogen is split off from pepsinogen upon its conversion into pepsin.

Experimental Procedure

The pepsin was dialyzed 24 hours at 10°C., first on the acid side of the isoelectric point against 0.02 N hydrochloric acid and then titrated to pH 4.5 and dialyzed 24 hours against M/1000 pH 4.65 acetate buffer. The pepsinogen was dialyzed 24 hours at 10°C. against M/1000 pH 6.8 phosphate buffer, then solid potassium sulfate was added to bring the solution to approximately 0.5 M. This was then dialyzed 24 hours at 10°C. against M/1000 pH 6.8 phosphate. These procedures were designed to remove or displace all split products and ammonium ion bound as the salt of the protein.

The formol titration was carried out by the procedure described by Northrop (15). 50–150 mg. of protein were used for each titration.

Denaturation was carried out by precipitating 50–150 mg. of protein with 5–10 volumes of the following reagents: 2.5 and 10 per cent trichloracetic acid; and by 0.5 M potassium sulfate. The protein was filtered or centrifuged and dissolved in dilute alkali. After solution of the precipitate the titration was carried out as usual except the solutions in which too much alkali was used to dissolve the precipitate; acid was added to bring them to pH 7.0.

In the Van Slyke gasometric procedure (14) the same amount of protein was used and denaturation was the same as in the formol titration experiments. After mixing the protein with the nitrous acid the chamber was shaken for 2 minutes, allowed to remain quiet for 23 minutes, and followed by a final shaking of 5 minutes.

Isoelectric Point

The isoelectric point of pepsinogen was determined by cataphoresis measurements (16) of pepsinogen coated collodion particles suspended in M/10 acetate buffers of various pH values. By this method an isoelectric point of 3.7 was obtained. Reproducible values of unactivated pepsinogen were obtained by working rapidly with dilute solutions of pepsinogen although activation takes place at this pH.

The isoelectric point was also determined by finding the pH at which either the minimal amount of alcohol precipitated pepsinogen or the maximum precipitate was obtained with a constant amount of alcohol. This work had to be carried out at 0°C. and as rapidly as possible to obtain a reading before appreciable activation took place. The breadth of the isoelectric region by these methods is certainly between pH 3.6 and 4.3 with 3.9 as the most likely isoelectric point. The isoelectric point of pepsin is pH 2.7 (5).

The pH measurements were made with a quinhydrone electrode standardized against known solutions of buffers.

Titration Curves

Complete titration curves of pepsin and pepsinogen might lead to a correlation of certain titratable groups with particular properties of these proteins. Such correlation would be of greatest interest in the region of the titration curve from pH 1.0–5.0 where pepsin is catalytically active and pepsinogen is inactive. In this range, however, the titration curves could not be obtained with the degree of precision necessary for such a correlation.

On the other hand, the titration curves of these two proteins could be determined at pH more alkaline than pH 4.0 and the differences noted correlated with certain properties, namely the stability of instability at given alkalinities. In Fig. 7 are the curves showing the amount of alkali combined per gram of protein.

Experimental Procedure

Solutions:

Pepsin.—2 times crystallized, dialyzed against M/1000 pH 4.65 acetate buffer and against 0.02 N hydrochloric acid followed by just enough alkali to dissolve. 7.5 mg. total nitrogen per ml.; 0.4 mg. non-protein nitrogen per ml., $[\text{P.U.}]^{\text{Hb}}_{\text{mg. P.N.}} =$ 0.25.

Pepsinogen.—Dialyzed, brought to 0.5 M with solid potassium sulfate, and dialyzed 24 hours to remove this salt and any ammonium ion. 6.3 mg. total nitrogen per ml., 0.0 mg. non-protein nitrogen per ml.

Procedures.—50 ml. of the pepsin plus 1 ml. of 5.0 N sodium chloride were introduced into the titrating chamber at 25°C. and the titration carried out with 5.0 N sodium hydroxide led into the solution from the capillary outlet of a microburette. The capillary tubing extended below the surface of the solution.

50 ml. of the pepsinogen solution plus 1 ml. of 5.0 N sodium chloride were cooled to 10°C. and mixed rapidly with 0.13 ml. of 5.0 N hydrochloric acid. This was

done to obtain the minimum activation and yet the lowest pH on the activation curve. Less than 5 per cent activation occurred. The pepsinogen solution was then warmed to 25°C. and poured into the titrating chamber and titrated with the same technique and alkali as was the pepsin. The electrodes were checked against M/10 pH 4.65 acetate buffer and read 0.515 volt. The solutions were made M/10 with respect to sodium chloride in order to raise the conductivity of the dialyzed protein solutions as well as to have the variation in ionic strength minimized during the experiment.

Apparatus.—The writer has found it convenient to run the titrations in an enlarged modified Clark rocking hydrogen electrode chamber. The chamber of 125 ml. capacity accommodates 50 ml. of solution nicely. The hydrogen passes over the solution and saturates it as it rocks without any appreciable foaming. The platinized electrode is in and out of the solution as the angle of the chamber changes. A fresh saturated potassium chloride contact is made through a three-way stop-cock stuffed with cotton to minimize diffusion. The arrangement is such that the saturated potassium chloride in the stop-cock is washed out in between each reading and replaced with fresh saturated potassium chloride.

Water Correction or Blank.—The correction for the quantity of alkali necessary to bring an equal volume of water to the various pH was experimentally determined with an equal volume of M/10 sodium chloride using the same alkali, apparatus, and technique as in the protein titrations. This avoids errors due to factors such as carbonate in the alkali, etc.

The pepsinogen titration curve II was started at the lowest pH at which reproducible values of pepsinogen could be obtained before activation proceeded to an appreciable extent. In the time required for the measurements of pepsinogen in the region of pH 4.4 there was less than 5 per cent activation. Curve I was started at the most acid pH at which the pepsin solution could be titrated without the protein precipitating from solution. Since the pH at which the titrations were started are not the isoelectric points of the two proteins curves I and II of Fig. 7 are not titration curves in the ordinary sense of the term. However, they do serve to show differences in the amount of alkali bound to the two proteins at various pH which in turn may be interpreted as meaning that there are more groups being titrated at a given pH in one protein than in the other.

Curve III is curve I plotted so that the value at pH 6.0 is the same as the pH 6.0 value of curve II. By so arranging them one can visually compare the slopes of the two curves at any pH.

It may be seen from curves II and III of Fig. 7 that between

pH 6.0 and pH 9.0 pepsin (curve III) binds 0.3 more millimols of alkali per gram of protein than does pepsinogen (curve II). In this region of alkalinity pepsinogen is unchanged, *i.e.* remains native, whereas pepsin is irreversibly denatured. Beyond pH 9.0 the pepsinogen curve rises sharply and approaches the pepsin curve at pH 12.0, indicating that pepsinogen has more groups titrating beyond pH 9.0 than does pepsin. Pepsinogen is also denatured by alkali but only beyond pH 9.0. This denaturation is, however, largely reversible (see section on Reversible alkaline denaturation). As may be seen in Fig. 7, curves II and III converge almost to a com-

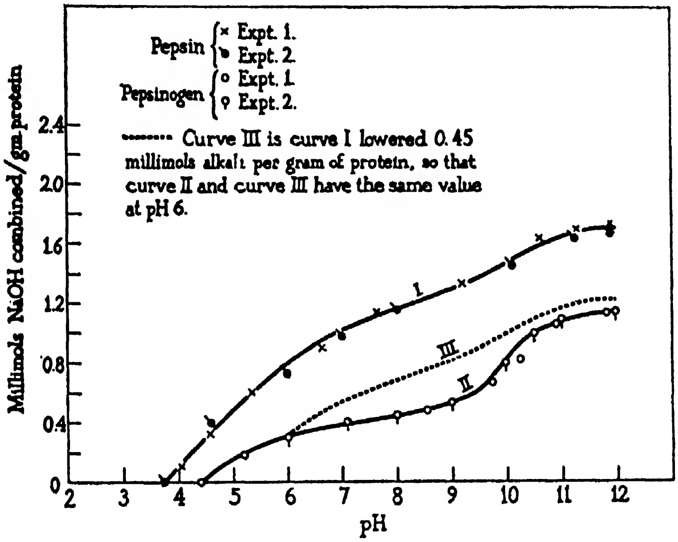

FIG. 7. Titration curves of pepsin and pepsinogen

mon point at pH 12.0. This means that the number of groups in pepsin and pepsinogen titrating between pH 6.0 and pH 12.0 is practically the same.

Molecular Weight

There must be a difference in the molecular weights of pepsinogen and pepsin and the former must be the larger for, as will be explained more fully in a later section of this paper, conversion of pepsinogen into the active enzyme results in two substances—pepsin and a fragment the nitrogen of which is 15–20 per cent of the pepsinogen nitrogen.

Experimental Procedure

The protein solutions plus the amount of ammonium sulfate to bring them to the desired concentration were placed in collodion bags and connected to mercury filled manometers. A marble inside the membrane insured constant stirring. The membranes were surrounded by 400–500 ml. of the corresponding salt solution and the units rocked slowly in a thermostatically controlled room at 10°C. Each figure in Table VIII is the result of at least three readings taken at 12–24 hour intervals after the equilibrium was established. The protein estimation was made by Kjeldahl nitrogen determinations. The factor 7.0 was used to convert nitrogen analyses into amount of protein.

TABLE VIII

Molecular Weight by Osmotic Pressure at 10°C. in Two Concentrations of Ammonium Sulfate Solution

Material	Salt concentration	Osmotic pressure	Protein/ml.	Molecular weight
	saturated	*mm. Hg*	*mg.*	
Pepsin	0.15	22	48	39,000
	0.15	23	48	37,000
	0.15	26	47	32,000
	0.30	20	46	41,000
	0.30	22	52	42,000
				38,000 ± 3,000
Pepsinogen	0.15	15	36	42,500
	0.15	17	36	37,500
	0.15	16	34	37,500
	0.30	17	45	47,000
	0.30	19	45	42,500
	0.30	19	48	45,000
				42,000 ± 3,000

The figures in Table VIII reveal that the molecular weights calculated from osmotic pressure measurements bear out the above conclusion. The precision of the osmotic pressure measurements is such that one cannot be certain about the actual difference in the molecular weights. However, the difference is in the right direction and of the right order of magnitude.

Tyrosine-Tryptophane Content

In view of the writer's previous work (13, 17, 18) on the relationship of tyrosine of pepsin to its enzymatic activity it seemed worth while

to see if the tyrosine of pepsinogen differed in content or reactivity from that in pepsin. When an estimation of the content was made with Folin's phenol reagent and the color allowed to develop at pH 8.0 there was a decided difference in the values of pepsin and of pepsinogen, as shown in Table IX. It has been pointed out by Mirsky and Anson (19) that the tyrosine phenol groups of denatured proteins react more readily with reducing agents than when the protein is native. In view of the fact that pepsinogen is native at pH 8.0 whereas pepsin is denatured, it was necessary to determine whether the difference in the amounts of these amino acids was due to a difference in the reactivity of the tyrosine, or to the fact that the conditions were not comparable; *i.e.*, the proteins were not both native or both denatured while being measured.

Experimental Procedure

Conditions for Color Development

pH 8.0.—The solutions, reagents, concentrations, etc., are similar to those previously described (17). One difference exists, namely that the 5 ml. of alkali-phosphate solution was mixed with the 3 ml. of Folin's phenol reagent before they were added to the protein solution. If the acid phenol reagent were added to the protein solution before the alkaline solution was added activation might have occurred in the case of pepsinogen. On the other hand, if the alkaline solution were added first it would have denatured the pepsinogen. By mixing them together and then introducing them into the protein solution the pH never got above or below pH 8.0 ± 0.2. The mixing must be rapid and even then occasionally a precipitate developed in the phenol reagent-alkaline phosphate mixture. When this occurred the sample was discarded for only clear solutions should be compared in a colorimeter.

pH 11.0–12.0.—To 19 ml. of a solution containing 1.5–2.0 mg. of protein was added 3 ml. of a one to three dilution of Folin's phenol reagent, followed by 3 ml. of 1.25 N sodium hydroxide solution. The solution at room temperature was read after 5 minutes against a similarly treated 0.15 mg. of tyrosine.

The results of the experiments on this point are given in Table IX. When the color reagent was applied in strong alkali, *i.e.* pH 11.0–12.0 or at pH 8.0 and 70°C., where pepsinogen is denatured, the values of pepsin and pepsinogen were nearly the same, showing that there is not an appreciable difference in the amount of the color giving amino acids present in the proteins but that the native and de-

natured forms of pepsinogen behave differently in their action with the phenol reagent. It has not been found possible to determine the reactivity of tyrosine in native pepsin and a comparison of the native proteins is therefore impossible from this point of view.

Optical Rotation

From the figures in Table X it appears that pepsinogen has a lower specific optical rotation than does pepsin. It also shows that crystalline pepsin from pepsinogen has the same optical rotation as crystalline pepsin prepared from the commercial products by the methods previously outlined (5).

TABLE IX

Tyrosine-Tryptophane Color Value (Expressed as Per Cent Tyrosine)

Conditions for color development		Pepsin	Pepsinogen (1)	Pepsinogen (2)
pH	Temperature			
	°C.			
8.0	35	5.0	3.2	3.2
8.0	70	5.6	5.1	5.5
11.0–12.0	25	9.3	8.8*	8.6*

* The explanation for the difference between these values and those at pH 8.0 has already been discussed (17).

TABLE X

Specific Optical Rotation of Crystalline Pepsins and Pepsinogens

Material	$[\alpha]^{D\ 25°C.}_{gm.\ dry\ weight}$
Crystalline Parke Davis pepsin	−72
Crystalline Cudahy pepsin	−71
Crystalline pepsin from pepsinogen	−72
Uncrystallized pepsinogen	−61
3 times crystallized pepsinogen	−62

Carbohydrate

The carbohydrate present in purified pepsin and pepsinogen preparations is less than 0.1 per cent which is less than 0.2 mol of hexose per mol of protein. It is therefore not to be considered as an intrinsic

part of the protein molecule. The methods used were essentially those of Sørensen and Haugaard (20) and of Elson and Morgan (21) the modification of the former being described under Experimental methods.

Ultraviolet Absorption Spectrum

Although the plotted curves of the ultraviolet absorption spectra of pepsin and pepsinogen are indistinguishable, nevertheless a consistent difference exists which may be recognized on the plates. One of the tyrosine bands in pepsinogen is different from that of pepsin. The writer is indebted to Dr. George I. Lavin for these measurements.

Serology

A detailed account of this subject has been given by Seastone and Herriott (3).

Conversion of Pepsinogen into Pepsin

When solutions of pepsinogen are made more acid than pH 6.0 the protein is converted into pepsin. This conversion has been studied from two different angles.

Kinetics of Conversion.—As stated in the preliminary report (22), the conversion of pepsinogen into pepsin is an autocatalytic reaction at pH 4.6. Fig. 8 shows graphically the analyses of a conversion experiment in which the activity was determined directly by the gelatin viscosity method $[\text{P.U.}]^{\text{Gel. V}}$ and indirectly by the hemoglobin method $[\text{P.U.}]^{\text{Hb}}$. The hemoglobin method as carried out measures the pepsinogen from which pepsin value is obtained by difference. It may be seen from Fig. 8 that the values by both methods fall reasonably close to the theoretical curve calculated from the average value of K using the simple autocatalytic formula

$$\frac{dA}{dt} = KA(A_e - A)$$

which after integration gives

$$K = \frac{2.3}{A_e t} \log\left(\frac{A}{A_e - A}\right)\left(\frac{A_e - A_0}{A_0}\right)$$

This equation also describes the conversion of trypsinogen into trypsin (23). A is the activity at time t, A_e is the final or equilibrium activity, and A_0 is the initial value of A or activity at zero time.

Experimental Procedure

To 35 ml. of a dialyzed solution of 3 times crystallized pepsinogen at pH 7.0 containing 1.4 mg. protein nitrogen per ml., was added 13.0 ml. of M/1 pH 4.5 acetate buffer. The entire system was kept at 25°C.

Hemoglobin Activity.—0.5 ml. aliquots of the activation mixture were added to 0.5 ml. of saturated (0.3 N) borax making the solution pH 8.0. This inactivates the pepsin present. After 5 minutes these solutions were acidified to pH 1.0–2.0 where they were allowed to activate completely for 10 minutes. Activity was then determined in the usual way by the hemoglobin method.

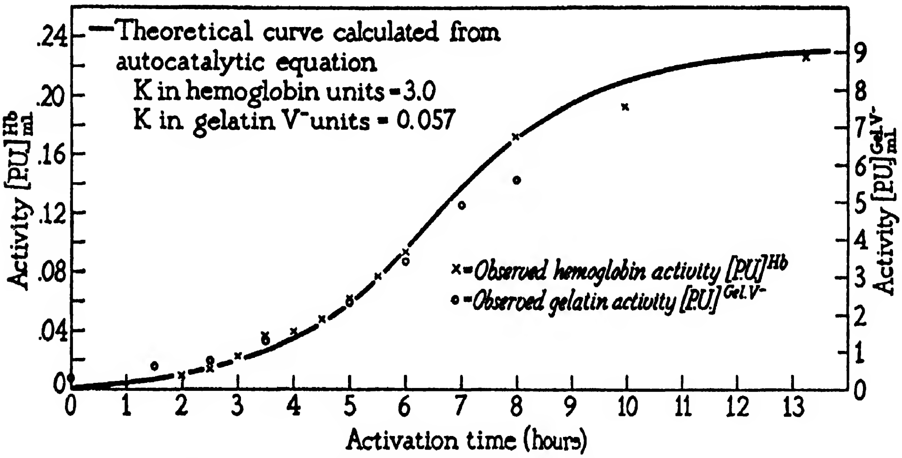

FIG. 8. Conversion of 1.0 mg. pepsinogen nitrogen per ml. into pepsin at pH 4.6 and 25°C.

Gelatin Activity.—The gelatin activity was determined directly on aliquots of the activation mixture by measuring the change in viscosity of an isoelectric (pH 4.7) gelatin solution as described by Northrop (12).

The autocatalytic constant K for the experiment in Fig. 8 in hemoglobin activity units is 3.0 ± 0.1, *i.e.* when 1.0 mg. of pepsinogen nitrogen per ml. is activated at pH 4.6 at 25°C. with a unit time as 1 hour and A expressed in hemoglobin activity units the fractional increase in A is 3.0 times per hour.

The conversion reaction rate varies enormously with pH. It is hardly perceptible at pH 6.0 while at pH 4.0 it is relatively rapid; *i.e.*, 1 mg. protein nitrogen per ml. at 25°C. activates in about 30

minutes. Near pH 1.0 where the rate is at a maximum it is of the order of 100 times the rate at pH 4.0. In solutions more acid than pH 4.0 the kinetics of activation appear to be not the simple autocatalytic reaction noted between pH 4.5–5.0 though it can be demonstrated that it is at least partially autocatalytic as far acid as pH 0.0. This is shown by an increase in conversion rate by the addition of pepsin.

Activation rates are greatly increased by the presence of salts. Kunitz and Northrop noted a similar increase by salt in the activation of trypsinogen (8). The increase in the rate of conversion of pepsinogen by salt seems to be a function of charge or valency of the ions of the salt as well as the concentration of the salt. A shift in pH by the salt explains part of the increased rate of activation but there is still a definite effect which appears to be related to the presence of the salt at least in the case of magnesium sulfate at pH 4.0.

Chemistry of Conversion.—When pepsinogen is changed into pepsin there is a concomitant production of non-protein nitrogen (nitrogen not precipitable by 2.5 per cent trichloracetic acid) to the extent of 15–20 per cent of the pepsinogen nitrogen. This is shown clearly in Fig. 9. Although the conversion was carried out under a number of different conditions of pH and temperature of which only two are shown the points all fall close to the same line in Fig. 9. That the line is straight in such a differential plot as A in Fig. 9 for two different sets of conditions and that the increase in non-protein nitrogen stops at the same time that the increase in activity stops, as shown in B of Fig. 9, seems to exclude the possibility of this phenomenon being a coincidental digestion of a small amount of protein impurity. It cannot be supposed that the increase in non-protein nitrogen is the complete digestion of part of the pepsinogen protein by pepsin since, as is shown in Table XI, the same amount of non-protein nitrogen is produced from a given amount of pepsinogen even though three times the quantity of pepsin is added at the start.

Experimental Procedure

pH 2.0 Experiment.—To 15.0 ml. of dialyzed pepsinogen at pH 6.8 containing 5.5 mg. protein nitrogen per ml. cooled to 0°C. was added 14.0 ml. N/10 hydrochloric acid and 11.0 ml. water both of which had been cooled. At definite intervals of time 4 ml. aliquots of this solution were added to 1.0 ml. of 0.3 N

borax bringing the pH to 8.5. After standing 5 minutes at room temperature 2.0 ml. aliquots of this alkaline solution were added to 10.0 ml. of boiling 2.5 per cent trichloracetic acid, cooled, filtered, and the nitrogen determined in the filtrate. The values in Fig. 9 were corrected to the original conditions of the reaction mixture. Another aliquot of the alkaline solution was added to hydrochloric acid of such concentration to bring the pH to 1.0–2.0 as indicated by thymol blue. Complete activation occurred within 5 minutes at room temperature. Activities were determined by the hemoglobin method. The value for 100 per cent activation was obtained by allowing an aliquot of the activation mixture to activate for 10 minutes at room temperature without going through the alkaline borax treatment.

pH 4.7 Experiment.—To 15.0 ml. of the same dialyzed pepsinogen solution was added 2.25 ml. of N/10 hydrochloric acid and 22.75 ml. of M/10 pH 4.65 acetate buffer. The solution was placed at 35.5°C. and at definite intervals of time 4.0

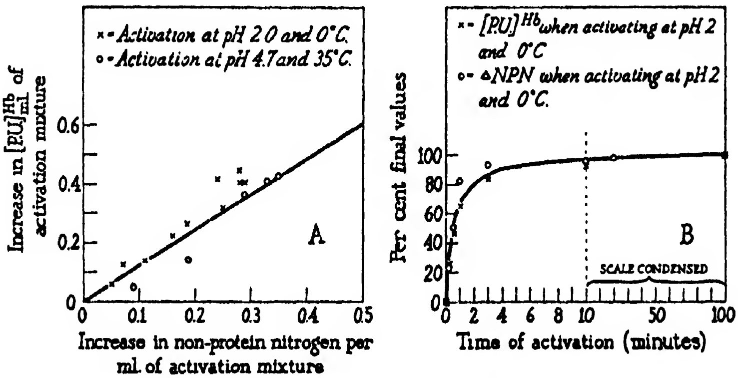

FIG. 9. Increase in non-protein nitrogen during activation at pH 2.0 and 4.7. *A*. Increase in non-protein nitrogen with increased activation. *B*. Rate of increase in non-protein nitrogen and hemoglobin activity.

ml. aliquots were removed and treated in an identical manner as in the above pH 2.0 experiment.

Experimental Procedure

Protein Solutions:

A.—Dialyzed 2 times crystallized Cudahy pepsin; pH 3.5; 10 mg. protein nitrogen per ml.

B.—Dialyzed 3 times crystallized pepsinogen, pH 7.0, 3.5 mg. protein nitrogen per ml.

First section of Table IX: 5.0 ml. *A* + 5.0 ml. *B*.
Second " " " " 5.0 ml. *B* + 5.0 ml. M/10 pH 4.0 acetate buffer.
Third " " " " 5.0 ml. *A* + 5.0 ml. M/10 pH 4.3 " " .

The final pH was practically 4.3 in all three sections. The solutions were kept at 35.5°C. and 2 ml. samples were added to 1 ml. of 0.3 N borax, bringing the pH

to 8.5 where it remained 5 minutes. 2.5 ml. of these alkaline solutions was then precipitated with 10 ml. of boiling 10 per cent trichloracetic acid and the filtrate analyzed for nitrogen. The remaining 0.5 ml. of alkaline mixture was activated with N/10 hydrochloric acid and analyzed by the hemoglobin method. The alkaline borax instantly inactivates the pepsin formed. Subsequent activation and analysis is a measure of the pepsinogen in the original activation sample.

Amino nitrogen analyses by the Van Slyke method show that upon conversion of pepsinogen to pepsin there is a total increase in amino nitrogen of not more than 0.3 per cent of the protein or 2.0 per cent of the total nitrogen which is equivalent to nine amino groups per

TABLE XI

Effect of Added Pepsin on the Increase in Non-Protein Nitrogen during the Activation of Pepsinogen

Materials	Activation time	Extent of activation	Increase in non-protein nitrogen
	min.	*per cent*	*mg./2 ml. reaction mixture*
Pepsin plus pepsinogen pH 4.3	0.33	37	0.11
	0.67	47	0.25
	1.00	61	0.21
	3.00	86	0.39
	420.00	—	0.63
Pepsinogen pH 4.3	2.00	35	0.18
	6.00	53	0.32
	15.00	84	0.41
	45.00	97	0.64
	420.00	—	0.52
Pepsin	420.00		0.00

molecule of protein. It is not known with certainty that the rupture of all nine of the linkages from which the amino groups were derived are involved or are necessary in the conversion.

Part, if not all, of the 15–20 per cent of the non-protein nitrogen after being produced as a result of conversion of pepsinogen into pepsin combines with pepsin between pH 5.0–6.0 to form a dissociable inhibitor-pepsin complex. That it inhibits pepsin is readily demonstrated by the rennet method which is carried out at pH 5.8. The inhibitor pepsin complex dissociates upon dilution and upon acidification. It does not combine with pepsin in acid solution so that it

does not affect the hemoglobin activity measurement but does eliminate the rennet method under certain conditions.

Experimental Methods

Preparation of Copper Hydroxide Suspension.—2,500 gm. of Merck's technical powdered copper sulfate is dissolved in 20 liters of tap water in a 50 liter battery jar. Into this solution, which is stirred mechanically, is run a solution made by mixing 800 ml. of saturated (19.0 N) U.S.P. sodium hydroxide and 15–20 liters of water. The suspension is thoroughly stirred and the precipitate then allowed to settle for 12–24 hours after which time the supernatant liquor is decanted away. The jar is again filled with tap water, stirred, the precipitate again allowed to settle, and the supernatant liquor discarded. The precipitate is now stirred up with 40 liters of distilled water and allowed to settle. After decanting off the clear water this precipitate is referred to as washed copper hydroxide. After the last decantation the precipitate is usually the correct concentration for use. Its exact concentration may readily be determined by treating an aliquot with enough sulfuric acid to dissolve it and comparing the resulting solution in a colorimeter with a solution of copper sulfate of known concentration. Several such analyses have shown the washed copper hydroxide to be about 1 molar in concentration.

Activity Measurements:

A. Estimation of Pepsin.—The solution is diluted and the activity determined as indicated below.

B. Estimation of Pepsinogen or Pepsinogen Plus Pepsin.—Acidify to pH 1.0–2.0, leave for 5 minutes at room temperature, dilute, and determine peptic activity.

C. Estimation of Pepsinogen Only in the Presence of Pepsin.[4]—Titrate or bring to pH 8.5 and leave for 5–10 minutes, acidify to pH 1.0–2.0 for 5 minutes at room temperature, dilute, and determine peptic activity.

D. Estimation of Pepsin Only in the Presence of Pepsinogen.—This can be carried out by measuring the peptic activity directly by the rennet method or by the difference in the hemoglobin activity of two aliquots treated as in *B* and *C*.

Hemoglobin.—This is the method of Anson and Mirsky (24).

Rennet.—The writer has combined several of the desirable features of the rennet methods of this and other laboratories into the following simple, rapid, and easily reproducible method.

"Klim" Solution.—20 gm. of Klim are worked into a paste in a mortar with water, poured into a 100 ml. graduate, and 10 ml. of M/1 pH 5.0 acetate buffer is added, finally diluting the suspension to 100 ml. with water. This solution should be kept in the ice box when not in use and for precise work it should not be used after 5 days from the time it is made up. A control tube should not clot in 24 hours at 35°C.

Procedure.—5.0 ml. of the above Klim solution is brought to 35.5°C. in a water bath and 0.5 ml. of the pepsin solution diluted in M/10 pH 5.0 acetate is added.

The pipette should be held about 1 inch above the Klim solution and the last drop blown out of the pipette. The test tube containing the digestion mixture is now twirled once or twice to mix the solutions and to wash down any enzyme solution which may be on the side of the tube. A stop-watch is started as the enzyme is added. The tube is now left in the bath until a minute or two before it should clot and then the tube is tipped and slowly rotated so that the worker can examine a thin film of the solution which thickens and coagulates in small particles just before clotting. The end point is arbitrary and therefore depends upon the worker. However, the variation in the end point determined by two workers is not great. The writer has chosen the first definite recognizable change in the film of milk as the end point. The stop-watch is, of course, stopped at the end point. Constant tipping or rotating of the tube has practically no effect on the clotting time.

Enzyme Solutions.—0.5 or 1.0 ml. of a solution of crystalline pepsin containing 0.003–0.0001 mg. nitrogen per ml. when added to 5 or 10 ml. of a 20 per cent Klim solution at 35.5°C. will clot it in 1–30 minutes. Over this range the time of clotting is inversely proportional to the pepsin concentration and does not vary with the time of standing in the acetate buffer. In activation mixtures of pepsinogen, on the other hand, the ratio of dilution to clotting time is not constant as with crystalline pepsin but increases with dilution. At any given dilution the activity also decreases with time of standing in the acetate buffer. This latter effect is more striking when the activation mixture is diluted in M/10 pH 5.6 acetate instead of the M/10 pH 5.0 buffer. This and other evidence indicates the presence of inhibiting material which, although dissociated in acid thus not affecting the hemoglobin method, combines with pepsin at pH 5.0–6.0 and reduces its rennet action. The inhibiting material may be separated from pepsin and on mixing with purified pepsin will give effects similar to those noted for activation mixtures. Crystallization of pepsin from an activation mixture leaves the inhibiting material in the mother liquor. It has recently been found that replacement of M/10 pH 5.0 acetate buffer by M/1 pH 5.0 acetate as the solvent for the Klim solution will greatly diminish these inhibitor effects; also that the activation mixture should be diluted in water and the aliquot added to the Klim solution immediately.

Rennet Activity Units $[P.U.]^{Ren.}$.—The writer has, for convenience, arbitrarily defined 1 rennet unit as the amount of enzyme which, under the above defined conditions, will clot 11 ml. of the enzyme-Klim mixture in 1 minute. This rennet unit is not to be confused with the previously described one (12). The rennet activity of any solution is obtained then by dividing the dilution by the time in minutes required to clot the Klim. Thus, if 1.0 ml. of an enzyme solution was diluted to 500 ml. and 1.0 ml. was added to 10.0 ml. of the 20 per cent Klim or 0.5 ml. to 5.0 with a clotting time of 5 minutes, the original solution would contain 500/5 or 100 $[P.U.]^{Ren.}_{ml.}$. The rennet activity per milligram of protein nitrogen $[P.U.]^{Ren.}_{mg. P.N.}$ for a number of different swine pepsin preparations is given in Table IV.

Carbohydrate Estimation.—The method of estimating carbohydrate used in these experiments was a modification of the Sørensen-Haugaard method (20). It is simple and rapid though not so precise or specific as the Sørensen-Haugaard scheme. The recent adaptation of their method by Heidelberger and Kendall (25) probably gives slightly more precise results than the writer's method which is as follows:

1.0 ml. of a solution of carbohydrate which will yield a color comparable to 0.1 mg. of glucose is introduced into a 50 ml. Erlenmeyer flask along with 15 ml. of 21.5 N sulfuric acid and 2.0 ml. of a 2 per cent solution of orcinol in 7.5 N sulfuric acid. The flask is placed in a stirred water bath at 80°C. ± 1.0° for 30 minutes after which it is cooled in cold water and read in an ordinary colorimeter against a similarly treated standard 0.1 mg. of glucose or against a solution of pepsinogen which had previously been standardized in terms of glucose. A green glass over the eye piece of the colorimeter aids in balancing the solutions. The color is stable for several hours and the writer has run a dozen or more samples at one time with ease and reproducibility.

Nitrogen Estimations.—The technique of running the Kjeldahls was that previously reported (11).

Protein nitrogens in the presence of appreciable amounts of ammonium sulfate were made by precipitating the protein from an aliquot with 5–10 volumes of boiling 5 or 10 per cent trichloracetic acid, cooling and filtering the precipitate, and washing it on the funnel with cold 2.5 per cent trichloracetic acid until the washings were free of ammonium ion as indicated by a negative Nessler test. The precipitate was then washed into a Kjeldahl flask and the nitrogen estimated as usual. With crude solutions of pepsinogen or in the presence of appreciable quantities of gastric mucin the protein nitrogens were very erratic due to incomplete precipitation or the solubility of the denatured material.

pH Estimation.—For the most part the pH determinations were colorimetric using the indicators recommended by Clark and Lubs (26). In certain cases hydrogen quinhydrone and glass electrodes were used but those cases are indicated in the procedures of the individual experiments. The values obtained by the use of indicators are the pH values of molar tenth buffers which give the same color.

SUMMARY

1. A method is described for the preparation of pepsinogen from swine gastric mucosae which consists of extraction and fractional precipitation with ammonium sulfate solutions followed by two precipitations with a copper hydroxide reagent under particular conditions. Crystallization as very thin needles takes place at 10°C., pH 5.0 and from 0.4 saturated ammonium sulfate solution containing 3–5 mg. protein nitrogen per milliliter.

2. Solubility measurements, fractional recrystallization, and fractionation experiments based on separation after partial heat or alkali denaturation and after partial reversal of heat or alkali denaturation failed to reveal the presence of any protein impurity.

3. The properties of the enzymatically inactive pepsinogen were studied and compared with the properties of crystalline pepsin. The properties of pepsinogen which are similar to those of pepsin are: molecular weight, absorption spectrum, tyrosine-tryptophane content, and elementary analysis. The properties in which they differ are: enzymatic activity, crystalline form, amino nitrogen, titration curve, pH stability range, specific optical rotation, isoelectric point, and the reversibility of heat or alkali denaturation.

4. Conversion of pepsinogen into pepsin at pH 4.6 was found to be autocatalytic; *i.e.*, the pepsin formed catalyzes the reaction. Conversion of pepsinogen into pepsin is accompanied by the splitting off of a portion of the molecule containing 15–20 per cent of the pepsinogen nitrogen.

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THE SUSCEPTIBILITY OF MICE TO INHALED TYPE III PNEUMOCOCCI

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In preceding papers[1] it had been shown that when mice are exposed to an atmosphere containing type I pneumococci in the form of a fine mist, the organisms readily penetrate into the lower respiratory tract. Pneumococci which have reached the lung as a result of this procedure usually disappear from the periphery of the lung within a few hours, and only in a few instances does death result from a septicemia. In mice intoxicated with alcohol, however, pneumococci persist in the lungs for a long period, and a fatal septicemia frequently follows. The purpose of the present paper is to report results obtained when mice were exposed to sprays containing type III pneumococci.

METHODS

Normal mice were allowed to inhale an atmosphere which contained a fine bacterial mist produced by spraying a stock culture of type III pneumococci which had been artificially cultivated for some years. The animals were placed in the spray chamber previously described and were sprayed with 50 cc. of broth culture. As a rule, the mice were removed from the spray box after an exposure of 1 hour. At various intervals following spraying, individual mice were sacrificed. In order to prevent the possibility of aspiration of bacteria from the upper to the lower respiratory tract at the time of death, the mice were killed instantaneously by clamping the trachea and spinal cord with sponge forceps. The mice were then immersed in a solution of lysol and autopsied with sterile instruments. From the mice which

1. J. Exper. Med. **38**: 117, 1923; **40**: 353, 1924.

were killed, small pieces of lung and a few drops of heart blood were separately cultured in plain broth. Only heart blood cultures were made from the mice that died. All positive cultures were plated on blood agar for further identification.

EXPERIMENTAL

In fig. 1 are shown the number of times pneumococci were recovered from the lungs of mice which were killed at stated intervals after the spraying of pneumococci into the air breathed by them. Each

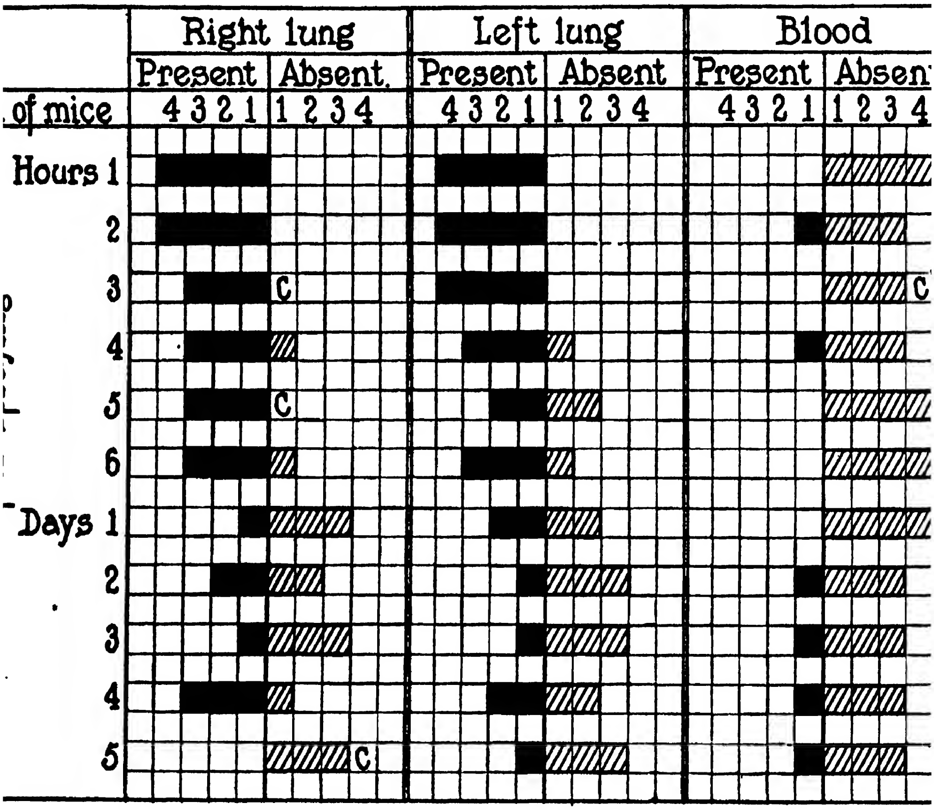

FIG. 1. The persistence of type III pneumococci in the lungs and blood of mice.

black square indicated one mouse from the lungs or heart blood of which pneumococci were recovered. The cross-hatched squares represent mice from which the test organisms were not recovered. In a few instances, especially in the cultures from the lungs, the identification of the sprayed organisms was not possible because of contami-

nation by other bacteria. All lung or blood cultures so contaminated are indicated in the figure by the letter C.

It is seen that while pneumococci were almost invariably recovered from the lungs within 6 hours after spraying, they were recovered only irregularly in the mice killed during the next 5 days.

Of the 44 mice sacrificed within 5 days, pneumococci were demonstrated in the blood in 6 instances. It is surprising to note that in mice killed at 2 and 4 hours respectively, pneumococci were recovered from the heart blood. This experiment demonstrates that when normal mice are allowed to breathe an atmosphere in which type III pneumococci are suspended, the inspired bacteria may persist in the lungs for a long period and may invade the tissues in sufficient numbers to cause septicemia.

TABLE 1

Number of Days Elapsing between Spraying and Death of Mice from Pneumococcus Septicemia

Days	2	3	4	5	6	7	8	9	11	12	14
No. of mice	10	32	50	29	17	11	10	2	2	1	1

In order to determine the incidence of infection, as expressed by death of mice following exposure to this strain of type III pneumococci, a number of experiments were performed, in which the technic was similar to that already described. In these experiments, however, the mice were not killed, but were kept under observation for a period of at least 14 days. Out of a total of 270 mice exposed, 166 or 54% died of pneumococcus septicemia. The interval elapsing between the time of spraying and death from pneumococcus septicemia is shown in table 1. From this it is seen that the greatest number of mice died within the first 5 days following exposure. Fewer died during the next 3 days, and an occasional mouse succumbed to septicemia after the 8th day.

Contact Infection

From the preceding experiments it is evident that type III pneumococci persist in the lungs of mice for a number of days following inhalation, and frequently cause a fatal septicemia. To learn whether

the organisms recovered represented a persistence of the bacteria originally acquired, or whether in some instances following the initial exposure mice became free of pneumococci to be reinfected later through contact, the following experiments were carried out. Ten mice were sprayed and immediately isolated in separated boxes. Six of these mice died of pneumococcus infection. Twenty other mice were similarly sprayed. Ten of the exposed mice were placed in one box. The other 10 mice were placed in single boxes to which were added 2 normal mice, one of which had been intoxicated by an injection of alcohol. In this experiment all 20 of the sprayed mice died of pneumococcus septicemia, but all the normal mice which had not been exposed by spraying survived. Contact infection did not seem to play a part in the infection which it would seem must have occurred at the time of spraying.

DISCUSSION

The results of these experiments support the previous observations that in mice exposed to a dense spray containing bacteria in suspension, the inhaled organisms penetrate to the smaller bronchi. The further history of the bacteria which have been implanted in the lungs by inhalation depends not only upon the kind, number and invasive quality of the bacteria, but also upon the normal defensive mechanism of the host. The variations in the number of bacteria gaining entrance to the respiratory tract following spraying are not subject to control. The ability of the host to resist infection may, however, be somewhat depressed by experimental procedures. Different degrees of invasiveness may be demonstrated by the use of different bacteria. The reaction of animals varies according to the invasive quality of different bacteria, different types of pneumococci, and even different strains of the same type of pneumococci.

It has been shown that pneumococci of types I and II, which are highly virulent for normal mice when given by intraperitoneal or subcutaneous inoculation, when implanted on the respiratory mucosa of normal mice by inhalation, disappear from the periphery of the lungs within a few hours and rarely give rise to infection. In order to cause invasion following spraying with type I or type II pneumococci, the normal resistive mechanism of the mouse must be depressed

by alcohol. In the case of the strain of type III pneumococci, used in this study, alcohol is not necessary because, following exposure of normal mice, the organisms may be recovered from them for days after the spraying, and frequently they may cause a fatal infection. It has also been shown that hemolytic streptococci and Friedländer's bacillus may be recovered from the lungs and heart blood for a number of days after exposure and a large number of animals so exposed die of a septicemia.[2]

Similar variations have been shown to exist in rabbits.[3] Whereas 37% and 28% of rabbits succumb following the first exposure to inhalations of type I and type II pneumococci respectively, 56% die after a single exposure to a rabbit virulent strain of type III pneumococci.

SUMMARY

The reaction of mice to inspired pneumococci varies according to the type of pneumococcus inspired.

Type I and type II pneumococci rapidly disappear from the lungs of normal mice and rarely cause septicemia.

Following inhalation of the strain of type III pneumococcus used in these experiments, the bacteria persisted in the lungs for several days and a fatal septicemia occurred in 54% of the exposed animals.

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DERIVATIVES OF GLUCURONIC ACID

VIII. THE STRUCTURE OF BENZOYLGLUCURONIC ACID

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When benzoic acid is ingested by dogs, it is excreted in the urine partly in the form of hippuric acid and partly as a benzoyl ester of glucuronic acid. The detoxication of aromatic organic acids by conjugation with glucuronic acid is one of the important physiological mechanisms of man and certain animals. Benzoylglucuronic acid was first described by Magnus-Levy (1) who isolated it from the urine of sheep in the form of a dextrorotatory crystalline strychnine salt, from which he prepared an amorphous sodium salt. The structure of 1-benzoylglucuronic acid was assigned to this compound, though a critical survey of the original communication of Magnus-Levy reveals no direct chemical evidence in support of this hypothesis. This explanation of the constitution of benzoylglucuronic acid has been generally accepted until recent years.

Some time ago the question of the structure of benzoylglucuronic acid was reopened by Quick (2) who obtained the compound from the urine of dogs as a crystalline levorotatory substance. It was observed that solutions of benzoylglucuronic acid, when treated with traces of alkali, underwent a rapid change in rotation, a phenomenon which was attributed to mutarotation. On the basis of this evidence and because benzoylglucuronic acid reacted with dilute sodium cyanide solution, it was suggested that the aldehydic carbon atom of the conjugated derivative is free and that the compound is an ester substituted not on the 1st, but on one of the remaining carbon atoms of the hexoseuronic acid. This postulation has been questioned by Pryde and Williams (3) who favor the 1-benzoyl structure for benzoyl-

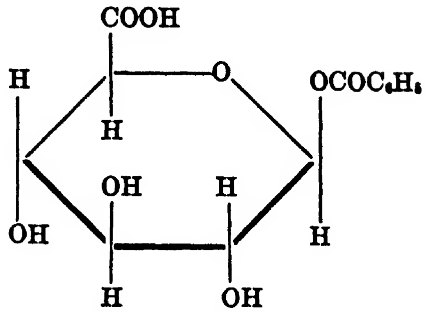
glucuronic acid. However, their interpretation of the experimental evidence in support of this contention has since been refuted by Quick (4). On the basis of the chemical evidence which is available at the present time it may be fairly stated that the exact constitution of benzoylglucuronic acid still remains obscure. It was thought possible, however, to establish the structure of this biologically important substance directly by synthesis.

The preparation of 1-bromo-2,3,4-triacetylglucuronic acid methyl ester has made possible the synthesis of derivatives of glucuronic acid substituted on the 1st or aldehydic carbon atom of the uronic acid (5). If benzoylglucuronic acid is an ester having the β configuration and substituted on carbon atom 1, as supposed by Magnus-Levy and by Pryde and Williams, then the triacetyl methyl ester of the naturally occurring derivative should be identical with the synthetic product obtained by condensing 1-bromo-2,3,4-triacetylglucuronic acid methyl ester with silver benzoate.

Natural benzoylglucuronic acid was isolated according to the method of Quick (2) and the methyl ester prepared by treating a cold alcoholic solution of the free acid with diazomethane. On acetylation of the latter derivative, triacetylmonobenzoylglucuronic acid was obtained in excellent yields. The substance crystallizes in the form of prismatic needles melting at 145° and having a specific rotation of −16.6° in chloroform. Synthetic 1-benzoyl-2,3,4-triacetylglucuronic acid methyl ester was secured by condensing 1-bromo-2,3,4-triacetylglucuronic acid methyl ester with silver benzoate in boiling chloroform. When recrystallized from methyl alcohol, the synthetic derivative was found to be identical with triacetylmonobenzoylglucuronic acid methyl ester prepared from natural benzoylglucuronic acid. The crystalline structure, specific rotation, analysis, and melting point of both derivatives are identical and a mixed melting point of the two substances shows no depression.

Since 1-bromo-2,3,4-triacetylglucuronic acid methyl ester is a pyranose derivative having the β configuration (6), the substitution product, 1-benzoyl-2,3,4-triacetylglucuronic acid, may likewise be assigned the same ring structure and configuration. The latter derivative, which has been synthetically prepared, is identical with that derived from natural sources. The parent substance, levorotatory benzoylglucuronic acid, may therefore be

assigned the following structural formula in which the benzoyl group is attached to the 1st carbon atom.



In view of the evidence presented above pertaining to the structure of benzoylglucuronic acid, it is unlikely that the change in rotation which solutions of the sodium salt of benzoylglucuronic acid undergo in the presence of sodium hydroxide or the *initial* rapid change in rotation of such solutions in the presence of excess sodium cyanide can be attributed to mutarotation as postulated by Quick (2). An alternative explanation is therefore suggested; namely, that the phenomenon is the result of a complex series of reactions initiated by a migration of the benzoyl group, catalyzed by hydroxyl ions, from the aldehydic to one of the remaining carbon atoms of the hexose-uronic acid. The migration of benzoyl and acetyl groups of partially acetylated sugars in the presence of hydroxyl ions is of course a well known phenomenon and it is not unlikely that the benzoyl radical of monobenzoylglucuronic acid also migrates in the presence of hydroxyl ions.

In conclusion it is suggested that the amorphous dextrorotatory sodium salt of benzoylglucuronic acid of Magnus-Levy is in all probability the rearranged form of the levorotatory derivative described by Quick, for the former substance was isolated from an alkaline urine which had stood about for a considerable period of time (1). When certain precautions are taken, however, as Quick has shown, crystalline levorotatory benzoylglucuronic acid can be isolated in excellent yields.

EXPERIMENTAL

Benzoylglucuronic Acid—This substance was prepared from the urine of dogs which had been fed benzoic acid according to the directions of Quick (2). Contaminating hippuric acid was re-

moved from the crude benzoylglucuronic acid by extraction with ether in a Soxhlet extractor. Because of exceptional laboratory facilities for working in the cold, excellent yields of benzoylglucuronic acid were obtained. The urine of two dogs fed 35 gm. of benzoic acid yielded 17.5 gm. of crude benzoylglucuronic acid. After several recrystallizations from water the derivative showed a melting point of 184–185° (uncorrected). $[\alpha]_D^{25} = -26.8°$ in H_2O ($c = 0.6$ per cent).

The specific rotation of an aqueous solution of the sodium salt of benzoylglucuronic acid prepared by adding 1 equivalent of 0.1 N NaOH to a weighed quantity of benzoylglucuronic acid at 0° was $[\alpha]_D^{30} = -27.7°$ ($c = 1$ per cent). This value is in marked contrast to that of the amorphous sodium salt of benzoylglucuronic acid reported by Magnus-Levy, $[\alpha]_D^{20} = +43.8°$.

Benzoylglucuronic Acid Methyl Ester—6.0 gm. of pure benzoylglucuronic acid were dissolved in 100 cc. of methyl alcohol and the solution cooled to −10°. An ethereal solution of diazomethane, also at −10°, was added in slight excess. The solvents were removed by distillation *in vacuo* and the residue dissolved in 300 cc. of boiling water. On cooling, crystals of benzoylglucuronic acid methyl ester separated. 4.5 gm. of substance were recovered. The compound melted at 190–191° (uncorrected).

$$[\alpha]_D^{25} = -16.3° \text{ in } CH_3OH \ (c = 1.5\%)$$

Analysis—$C_{12}H_{13}O_6COOCH_3$. Calculated, OCH_3 9.9; found, 10.1

Quick reported a melting point of 178–180° and $[\alpha]_D^{20} = -25°$ in H_2O for this derivative (2).

Acetylation of Glucuronic Acid Methyl Ester—3.0 gm. of benzoylglucuronic acid methyl ester were dissolved in a mixture of 12.5 cc. of freshly distilled anhydrous pyridine and 8.5 cc. of acetic anhydride at −5°. After standing for 1½ hours at 0° the mixture was gradually warmed to room temperature. The solution remained clear and colorless. The solvents were removed by distillation *in vacuo* and the residue dissolved in 25 cc. of methyl alcohol. 3.7 gm. of prismatic needles melting at 143–145° were recovered.

After several recrystallizations from methyl alcohol the compound melted sharply at 145° (uncorrected).

$$[\alpha]_D^{28} = -16.6° \text{ in } CHCl_3 \ (c = 1.5\%)$$

Analysis—$C_{20}H_{22}O_{11}$. Calculated. C 54.8, H 5.1, OCH_3 7.1
Found. " 55.1, " 5.3, " 7.1

Synthesis of 1-Benzoyl-2,3,4-Triacetylglucuronic Acid Methyl Ester—2.0 gm. of 1-bromo-2,3,4-triacetylglucuronic acid methyl ester were dissolved in 50 cc. of anhydrous alcohol-free chloroform and 3.5 gm. of anhydrous silver benzoate added. The mixture was refluxed for 3½ hours, filtered, and the chloroform evaporated from the filtrate *in vacuo*. The colorless syrupy residue was dissolved in 15 cc. of methyl alcohol. Crystals of monobenzoyltriacetylglucuronic acid methyl ester separated immediately. After standing at 0° for 24 hours, 1.7 gm. of substance were filtered from the mother liquors. After several rapid recrystallizations from methyl alcohol, 1.4 gm. of substance melting at 145° were secured. $[\alpha]_D^{28} = -16.9°$ in $CHCl_3$ (c = 1.5 per cent).

When monobenzoyltriacetylglucuronic acid methyl ester is crystallized slowly from methyl alcohol or from ether, the derivative is obtained in the form of large rhombs. The melting point of this crystalline form is apt not to be as sharp as that obtained when the derivative is crystallized rapidly from methyl alcohol.

Synthetic 1-benzoyl-2,3,4-triacetylglucuronic acid methyl ester is identical with the triacetyl ester derivative prepared from natural benzoylglucuronic acid as described above. A mixed melting point of the two substances showed no depression. The crystalline structure of the two preparations was identical and the specific rotations were likewise the same.

SUMMARY

1. The synthesis of 1-benzoyl-2,3,4-triacetylglucuronic acid methyl ester and its preparation from natural benzoylglucuronic acid are described.

2. The structure of benzoylglucuronic acid has been ascertained.

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ANTIPNEUMOCOCCUS RABBIT SERUM AS A THERAPEUTIC AGENT IN LOBAR PNEUMONIA

II. ADDITIONAL OBSERVATIONS IN PNEUMOCOCCUS PNEUMONIAS OF NINE DIFFERENT TYPES

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The use of type specific antipneumococcus rabbit serum as a therapeutic agent in lobar pneumonia has been reported in previous papers.[1,2] The various theoretical advantages of rabbit antiserum, the rationale which formed the basis for its clinical trial, and the results obtained in twenty-two cases of lobar pneumonia resulting from infection with pneumococcus Types I, II, VII, or VIII have been presented.

Since the publication of these initial papers additional experience has been gained in the use of antipneumococcus rabbit serum. Up to the present time sixty-seven patients have been treated with type specific antipneumococcus rabbit sera prepared in this laboratory.

It is the purpose of this paper to present the results obtained in the entire group of sixty-seven cases of lobar pneumonia which have been treated with type specific antipneumococcus rabbit serum. The group includes forty-five cases of lobar pneumonia which have not been previously reported, as well as the twenty-two cases described in an early paper.[2] Nine different types of pneumonia—I, II, III, V, VI, VII, VIII, XIV, and XVIII—have so far been treated with unconcentrated homologous antipneumococcus rabbit serum.

Methods

Antisera: Type specific antipneumococcus rabbit sera have been prepared and tested according to the technic described in a previous paper.[3] It has been found

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to be desirable to heat the antiserum to 56°C. for thirty minutes, and subsequently to absorb it with sterile kaolin for fifteen hours at 4°C. in order to reduce chill producing substances. It has also been found necessary to test the antiserum after it has been treated in the manner described, by the intravenous injection of two c.c. in each of three normal rabbits, according to the technic described[3] in order to estimate the reduction in chill producing substances. As was indicated previously[3] there is a very close correlation between the mean thermal reaction produced in normal rabbits and the extent of the chill reaction caused in human beings by the intravenous injection of antipneumococcus rabbit serum. When antisera which had been treated by the method described did not cause a significant thermal reaction in rabbits, no chill reaction followed the intravenous injection of these antisera in patients with lobar pneumonia. This correlation will be more fully discussed below.

Cases: The cases in this series have been an unselected group of patients with lobar pneumonia. In so far as it has been possible, the clinical study and the care of the patients has been identical with that outlined in the previous paper.[2] A complete physical examination was made immediately after admission to hospital. The physical signs indicating consolidation of one or more lobes of the lung were confirmed by x-ray examination. The pneumococci in the sputum were typed by the Neufeld technic and subsequently the type was confirmed by mouse inoculation. Blood cultures were taken shortly after admission and repeated as frequently as was desirable. As soon as the type of pneumococcus in the sputum had been determined, tests for sensitiveness to rabbit serum were carried out.

Tests for Sensitiveness

1. Intradermal test: The initial experience concerning the frequency with which falsely positive skin tests are encountered after the intradermal injection of .1 c.c. of a 1:10 dilution of normal rabbit serum into human beings,[2] has been confirmed by subsequent testing. No reliance has been placed on the results of the intradermal test because of the commonness of false positive reactions when rabbit serum is used. A markedly positive test occasionally occurs in patients subsequently shown not to be sensitive to rabbit serum on intravenous injection. It is believed, on the basis of preliminary tests, that the number of false positive reactions can be reduced by substituting normal serum diluted 1:100.

2. Conjunctival test: This test is carried out by instilling 0.1 c.c. of a 1:10 dilution of normal rabbit serum into one conjunctival sac. While this test has been of little aid in forming judgment as to sensitivity, a positive reaction would be taken as an indication that all subsequent procedures should be undertaken with the greatest caution.

3. Intravenous test: The intravenous test, however, has been found to be entirely satisfactory and no patient in the entire series of sixty-seven patients has been found to show any evidence of sensitiveness to rabbit antiserum in whom the intravenous test was negative. In four instances, more fully described below, both the clinical history and previous tests with *horse* serum indicated marked sensitive-

ness to the serum of this species. In two of these four cases the intravenous test with rabbit serum was entirely negative, and in both of these patients a full therapeutic dose of antiserum was given in one injection without the production of untoward symptoms. In the other two cases, however, the intravenous test with rabbit serum was strongly positive. Despite this fact, it was possible to give a full therapeutic dose of rabbit antiserum intravenously by beginning very slowly with extremely dilute antiserum, and with great caution gradually increasing the concentration of the serum injected.

For the intravenous test for sensitiveness it has been the routine practice to inject .1 c.c. of the therapeutic rabbit antiserum to be administered, diluted to five c.c. with physiological saline. Although this quantity of serum injected intravenously as a test dose, caused no reaction in sixty-five patients, it was large enough to produce a moderately severe reaction in the two patients who gave definite histories of allergy and were known to be sensitive to horse serum. It would undoubtedly be safer, as a routine, slowly to inject intravenously .05 c.c. of rabbit antiserum diluted to five c.c. with physiological saline. In cases where a suggestive history of allergy is elicited, or where hypersensitiveness to the serum of another species is demonstrated, it would seem wise to inject not more than .01 c.c. of rabbit antiserum diluted to five c.c.

A positive reaction to the intravenous test is manifested by a decrease in the arterial systolic pressure of fifteen mm. of Hg. or more, and an increase in the cardiac rate of fifteen beats per minute or more, both occurring within five minutes or less after the injection of the test dose of antiserum. The test, under the conditions stated, gives distinct and sharply defined results. In sixty-five cases the intravenous test was entirely negative, that is there was either no change in the arterial pressure and the cardiac rate, or the alterations in both were so slight as to be insignificant. On the other hand, in two cases the test was markedly positive as manifested by the usual rapidly occurring and dramatic signs of mild shock, accompanied by a sharp fall in arterial pressure and a marked rise in cardiac rate. In none of the sixty-five patients in whom the intravenous test was negative did there develop symptoms suggesting any sensitiveness to rabbit serum during or after the administration of the full therapeutic dose.

Administration of antiserum: One half hour after the intravenous test for sensitiveness to rabbit serum had been shown to be negative, the therapeutic administration of antiserum was begun. Undiluted antipneumococcus rabbit serum has been given intravenously throughout these studies. The rate of injection of the antiserum has been quite rapid in most instances. One hundred and twenty c.c. of antiserum may be injected safely intravenously in ten minutes. As much as three hundred c.c. have been given in this short period without producing discomfort. In the last fifty cases an effort has been made to give an adequate therapeutic quantity of antiserum in a single injection. It has been found that the oral administration of acetyl salicylic acid just prior to the injection of rabbit antiserum, as was previously suggested,[2] is advisable even if the antiserum has been shown by intravenous tests in rabbits to be free of significant amounts of chill producing substance.

Of the sixty-seven cases of lobar pneumonia which have been treated with unconcentrated type specific antipneumococcus rabbit serum prepared in this laboratory, thirty-nine have been treated in the Hospital of the Rockefeller Institute, sixteen in the Harlem Hospital, six in the Babies Hospital (New York City), two in the Albany Hospital, Albany, N. Y., and one each in the New York Hospital, the Presbyterian Hospital (New York City), the Plainfield Hospital, N. J., and the Middletown Hospital, N. Y.*

Because lobar pneumonia is not one disease, but a group of specific infectious diseases,[9] each of which has a different etiology and a different specific treatment, it is important to consider the patients treated with type specific antipneumococcus rabbit serum in groups according to the etiology of the infection.

Type I

Twenty-five patients with Type I pneumonia have been treated with unconcentrated Type I antipneumococcus rabbit serum. This group includes the ten patients with Type I pneumonia previously reported.[2] In Table I are presented the chief facts concerning this group of cases with lobar pneumonia. It will be observed that of the twenty-five, only four were females.

The average age of the group was thirty-three years, and included in the series are two children aged six and eight years respectively. Consolidation of two or more lobes occurred in nine cases, and in four cases it was bilateral. Type I pneumococcus bacteremia was present in eleven cases. Pleural exudates infected with Type I pneumococci were encountered in two cases. Type I antipneumococcus rabbit serum therapy was begun on an average of seventy-seven hours after the onset of the disease; in two cases it was delayed for 220 and 330 hours respectively.

* We wish to express our sincere appreciation for the courtesy and generous co-operation that has been shown us by the staffs of these various institutions. We are particularly grateful to Dr. Harriet Alexander, Dr. Richard T. Beebe, Dr. Jesse G. M. Bullowa, Dr. John B. Caffey, Dr. Howard R. Craig, Dr. Yale Kneeland, Jr., Dr. Rustin McIntosh, Dr. Thomas Ordway, Dr. Dan Witt, Dr. Arthur W. Wright, and Dr. F. Howell Wright for their kindness in allowing us to publish the results of the treatment of their cases with unconcentrated antipneumococcus rabbit serum.

Four patients in this series had been found to be extremely hypersensitive to *horse* serum. In two of these four patients there was no evidence of hypersensitiveness to rabbit serum, while the other two were markedly hypersensitive to both horse and rabbit serum. Even in the latter two cases, however, it was possible to give full therapeutic quantities of rabbit serum intravenously in a relatively short period of time. This was accomplished by the following technic: Into the tube of a continuously flowing glucose-saline intravenous infusion increasing quantities of diluted rabbit antiserum were injected. The initial rate of .0001 c.c. of antiserum per minute was doubled every ten minutes, and in this way within a period of three hours the full quantities of antiserum were given without any reaction.

In the whole group a mean quantity of 134 c.c. of unconcentrated Type I antiserum was injected intravenously per case. The average time which elapsed between the intravenous test dose and the completion of serum therapy in the last twenty-two cases has been but five hours.

The acute signs of the disease, as judged by the temperature, pulse and respiratory rates, have disappeared on the average nineteen hours after the institution of serum therapy. Thirteen cases received an adequate therapeutic quantity of serum in a single intravenous injection and in this group the average period from the beginning of serum therapy until the completion of crisis was but seven hours.

The two patients with infected pleural exudates must be considered separately. Both of these cases had Type I pneumococcus bacteremia. The first was a male of fifty-seven years. Serum therapy was commenced on the third day of the disease. Type I antibody of rabbit origin became demonstrable in the pleural exudate, and subsequently the pneumococci disappeared. Empyema did not develop. The second patient was a male, aged six. Serum therapy was commenced on the ninth day of the disease. The infection of the pleural exudate was not controlled and empyema necessitating surgical drainage developed. Serum sickness occurred in two cases.

Twenty-four cases recovered and one died, a mortality rate of four per cent. The single death in the series occurred in a male aged fourteen, three hours after the onset of an extremely severe chill reaction.

Type II

Ten patients with Type II pneumonia have been treated with unconcentrated Type II antipneumococcus rabbit serum. This group

TABLE I

Type I Pneumonia Treated with Type I Antipneumococcus Rabbit Serum

Case number	Sex	Age	Lobes consolidated	Bacteremia	Infected pleural exudate	Serum hours after onset	Serum c.c.	Crisis hours after serum	Result*
1	M	57	3	+	+	63	112	95	R
2	F	36	3	+	0	101	115	24	R
3	M	30	2	+	0	15	220	39	R
4	M	8	1	0	0	39	32	12	R
5	M	54	1	+	0	44	160	10	R
6	M	29	2	+	0	47	117	15	R
7	M	46	1	0	0	30	140	14	R
8	F	39	1	+	0	34	140	14	R
9	M	43	1	+	0	35	90	6	R
10	F	50	1	+	0	14	120	5	R
11	M	43	1	0	0	36	120	8	R
12	M	25	1	0	0	120	120	8	R
13	M	16	1	0	0	28	118	2	R
14	F	23	1	+	0	108	120	8	R
15	M	38	2	+	0	52	340	42	R
16	M	29	1	0	0	100	120	4	R
17	M	38	1	0	0	76	120	4	R
18	M	23	1	0	0	54	120	4	R
19	M	56	1	0	0	52	100	8	R
20	M	6	3	+	+	220	72	98	R
21	M	14	1	0	0	76	80	—	D
22	M	47	2	0	0	150	140	8	R
23	M	14	2	0	0	60	120	6	R
24	M	29	2	0	0	330	120	20	R
25	M	21	1	0	0	50	315	34	R
Average	..	33	..	..	..	77	134	19	..
Per cent	..	..	..	..	..	...	...	..	4

* R indicates recovery. D indicates death.

includes the four patients with Type II pneumonia previously reported.[2] In Table II are presented the salient facts concerning this group of cases. It will be noted that of the ten patients, three were

females and seven were males. The average age of the group was forty years. Consolidation of two or more lobes occurred in three cases. Type II pneumococcus bacteremia was present in one case. Pleural exudates infected with Type II pneumococcus were encountered in two cases.

Type II antipneumococcus rabbit serum therapy was commenced on an average of sixty-eight hours after the onset of the disease. It seems important to point out that two cases in this series were in the

TABLE II

Type II Pneumonia Treated with Type II Antipneumococcus Rabbit Serum

Case number	Sex	Age	Lobes consolidated	Bacteremia	Infected pleural exudate	Serum hours after onset	Serum c.c.	Crisis hours after serum	Result*
1	F	60	1	+	+	50	229	48	D
2	F	36	1	0	0	52	155	80	R
3	M	19	2	0	+	49	268	68	R
4	M	50	2	0	0	145	210	6	R
5	M	27	1	0	0	30	200	10	R
6	F	40	1	0	0	80	450	60	R
7	M	42	1	0	0	146	252	14	R
8	M	46	1	0	0	76	222	24	R
9	M	42	2	0	0	3	220	72	R
10	M	35	1	0	0	48	42	24	R
Average	..	40	..	..	..	68	225	41	..
Per cent	..	...	..	..	..	...	...	..	10

* R indicates recovery. D indicates death.

fourth day of the disease, while two other cases were in the sixth day when Type II rabbit serum therapy was begun. A mean quantity of 225 c.c. of unconcentrated Type II antiserum was injected intravenously per case. The acute signs of the disease, as indicated by the temperature, pulse and respiratory rates, disappeared on an average of forty-nine hours after the institution of serum therapy. Serum sickness occurred in six cases.

The two patients with infected pleural exudates must again be considered individually. In one patient, a male of nineteen years, serum therapy was begun on the third day of the disease. Type II

antibody of rabbit origin became demonstrable in the infected exudate and subsequently the pneumococci disappeared. Empyema did not occur. In the other patient, a female of sixty years, serum treatment was started on the third day of the disease. The infected exudate did not become sterile and empyema, necessitating surgical drainage, developed.

Nine cases recovered and one died—a mortality rate of ten per cent. The single death in this series occurred five weeks after the onset of the disease and resulted from the rupture of an aneurysm of the thoracic aorta into the empyema cavity.

Type III

Thirteen patients with Type III pneumonia have been treated with unconcentrated Type III antipneumococcus rabbit serum. In Table III are presented the results obtained in this group of cases. Of the thirteen patients, three were children aged two, five, and six years. All three children had bilateral Type III otitis media. The average age of the ten adult cases was fifty-four years. Among the thirteen cases there were seven with consolidation of two or more lobes. Four cases had Type III pneumococcus bacteremia. One pleural exudate infected with Type III pneumococcus was encountered. Auricular fibrillation occurred in four patients, and eight were sufficiently ill to require oxygen therapy.

Serum therapy was instituted on an average of fifteen hours after the onset of the disease. A mean quantity of 320 c.c. of unconcentrated Type III antipneumococcus rabbit serum was given to the ten adult cases, and 129 c.c to the three children. In those who recovered, the acute signs of the disease disappeared at an average of seventy-nine hours after the beginning of serum therapy. Serum sickness occurred in six patients.

Seven patients recovered, and six patients died—a mortality rate of forty-six per cent. The three children were among the patients who recovered. One adult female of fifty-two years, who had Type III pneumococcus bacteremia, also recovered.

Two patients deserve special attention. The first was a male of forty-five, who besides being alcoholic was admitted in a moribund condition on the seventh day of the disease. This patient had

Type III pneumococcus bacteremia and empyema, and lived for only eighteen hours after admission. The second was a female of seventy-two, who, eighteen hours after the injection of 281 c.c. of antiserum sustained a critical drop to normal in temperature, pulse and respiratory rates. This state was maintained for thirty-eight hours, at the end of which time the blood pressure rose sharply to 220/140. The patient had been known to have had hypertension prior to the development of pneumonia. Shortly after the sudden rise in blood pressure there developed signs of an intracerebral hemorrhage and death promptly followed.

TABLE III

Type III Pneumonia Treated with Type III Antipneumococcus Rabbit Serum

Case number	Sex	Age	Lobes consolidated	Bacteremia	Infected pleural exudate	Serum hours after onset	Serum c.c.	Crisis hours after serum	Result*
1	M	2	1	0	0	84	115	40	R
2	M	5	1	0	0	96	123	104	R
3	M	45	1	+	+	170	221	—	D
4	M	53	3	+	0	100	355	—	D
5	F	6	2	0	0	160	150	12	R
6	F	58	3	0	0	36	260	—	D
7	F	52	1	+	0	103	390	39	R
8	F	72	2	0	0	48	281	—	D
9	M	35	1	0	0	25	75	16	R
10	F	60	2	0	0	52	240	—	R
11	F	57	3	0	0	48	482	300	R
12	F	49	1	0	0	18	385	47	R
13	F	64	3	+	0	42	508	—	D
Average	..	54	..	..	..	75	320	79	..
Per cent	..	..	..	..	..	...	...	..	46

* R indicates recovery. D indicates death.

Type V and VI

One case of Type V pneumonia and two cases of Type VI have been treated with unconcentrated homologous antipneumococcus rabbit serum. The chief findings in these three cases are shown in Table IV. The ages of these three patients were two years, thirteen,

and fifteen months respectively. All had consolidation of two or more lobes. Both cases with Type VI pneumonia had Type VI pneumococcus bacteremia. In two cases, one of Type V and the other of Type VI, homologous pneumococcus empyema was present before serum therapy was started, and in the Type VI case the empyema was bilateral. In the other Type VI case there was a pleural exudate infected with Type VI pneumococcus. In each instance antipneumococcus rabbit serum therapy was commenced very late in the disease. The interval which had elapsed from the onset of the disease until serum was injected was thirteen days, five, and nine weeks respectively. The average quantity of unconcentrated rabbit antiserum which was

TABLE IV

Type V Pneumonia Treated with Type V Antipneumococcus Rabbit Serum

Case number	Sex	Age	Lobes consolidated	Bacteremia	Infected pleural exudate	Serum weeks after onset	Serum c.c.	Crisis days after serum	Result*
1	M	2	2	0	+	2	190	4	R
Type VI Treated with Type VI Antipneumococcus Rabbit Serum									
1	M	13m	3	+	+	5	50	1	R
2	M	15m	2	+	+	9	100	1	R

* R indicates recovery.

given intravenously was 113 c.c. per case. After the administration of antiserum bacteremia disappeared in both Type VI cases and there was a striking clinical improvement within twenty-four hours. In the Type V case there was as definite but less rapid improvement. Serum sickness did not develop in any of these cases. Both of the patients in whom empyema was present before serum was administered, underwent rib resection and surgical drainage of the infected pleural cavity. In the other case, in which the pleural exudate was infected with Type VI pneumococcus, the pneumococci disappeared from the exudate after the administration of antiserum and empyema did not develop. All three cases recovered.

Type VII

Six patients with Type VII pneumonia have been treated with unconcentrated Type VII antipneumococcus rabbit serum. This

group includes three patients with Type VII pneumonia previously reported.[2] The more important facts concerning this group of cases are shown in Table V. The average age in this group was forty-eight. One patient had consolidation of two lobes, and another had Type VII pneumococcus bacteremia. One patient developed auricular fibrillation.

On an average, serum therapy was commenced fifty-five hours after the onset of the disease. In two patients serum was not administered until the fourth day of the disease. A mean quantity of 160 c.c. of unconcentrated Type VII rabbit antiserum was injected intravenously per case. The acute signs of the disease disappeared on an average of seventeen hours after the institution of serum therapy. Two patients received an adequate therapeutic quantity of antiserum in a single injection. In these two cases the periods from the administration of serum to the completion of crisis were nine and eight hours. Serum sickness developed in five. All six recovered.

TABLE V

Type VII Pneumonia Treated with Type VII Antipneumococcus Rabbit Serum

Case number	Sex	Age	Lobes consolidated	Bacteremia	Infected pleural exudate	Serum hours after onset	Serum c.c.	Crisis hours after serum	Result*
1	M	35	1	+	0	37	164	39	R
2	M	32	1	0	0	34	122	11	R
3	M	60	1	0	0	28	150	9	R
4	F	68	2	0	0	34	224	40	R
5	F	54	1	0	0	98	164	9	R
6	F	39	1	0	0	100	140	8	R
Average	..	48	..	..	..	55	160	17	..
Per cent	..	..	..	..	..	...	...	..	0

* R indicates recovery.

Type VIII

Seven patients with Type VIII pneumonia have been treated with unconcentrated Type VIII antipneumococcus rabbit serum. This group includes five with Type VIII pneumonia previously reported.[2] The chief findings in this group of cases are shown in Table VI. The average age of the group was forty. Consolidation of two lobes

occurred in two patients, and Type VIII pneumococcus bacteremia occurred in two other patients. Serum therapy was begun on an average of thirty-five hours after the onset of the disease. A mean quantity of 160 c.c. of unconcentrated Type VIII rabbit antiserum was injected intravenously per case. The acute signs of the disease disappeared on an average of nineteen hours after the institution of serum therapy. Three cases were given an adequate therapeutic quantity of antiserum in a single injection and in these cases the average interval from the administration of serum to the completion of crisis was nine hours. Serum sickness developed in four. All seven recovered.

TABLE VI

Type VIII Pneumonia Treated with Type VIII Antipneumococcus Rabbit Serum

Case number	Sex	Age	Lobes consolidated	Bacteremia	Infected pleural exudate	Serum hours after onset	Serum c.c.	Crisis hours after serum	Result*
1	F	52	1	+	0	23	185	33	R
2	M	24	2	0	0	26	267	43	R
3	M	15	1	0	0	74	135	19	R
4	F	56	1	+	0	29	150	12	R
5	F	68	1	0	0	40	127	12	R
6	M	32	1	0	0	20	120	8	R
7	M	33	2	0	0	33	140	7	R
Average	..	40	..	..	..	35	160	19	..
Per cent	..	..	..	..	..	..	...	..	0

* R indicates recovery.

Type XIV and XVIII

Two patients with Types XIV and one with Type XVIII pneumonia have been treated with unconcentrated homologous antipneumococcus rabbit serum. The salient facts concerning these cases are shown in Table VII. The two patients with Type XIV pneumonia were fifty-three and twenty-five years of age respectively. The patient with Type XVIII was twenty-one years of age and was sufficiently ill to require oxygen therapy. All three patients had consolidation of but one lobe. Bacteremia was not present in any of the cases. In the cases with Type XIV, serum therapy was begun on

TABLE VII

Type XIV Pneumonia Treated with Type XIV Antipneumococcus Rabbit Serum

Case number	Sex	Age	Lobes consolidated	Bacteremia	Infected pleural exudate	Serum hours after onset	Serum c.c.	Crisis hours after serum	Result*
1	M	53	1	0	0	28	360	68	R
2	M	25	1	0	0	104	180	40	R
Type XVIII Treated with Type XVIII Antipneumococcus Rabbit Serum									
1	M	21	1	0	0	48	332	24	R

* R indicates recovery.

TABLE VIII

Comparison of Incidence of Chill Reactions Following Injection of Untreated and Treated Antipneumococcus Rabbit Serum

Antipneumococcus Rabbit sera	Number of lots	Patients			Injections		
		Number	Number having chills	% having chills	Number	Number producing chills	% producing chills
Untreated	14	31	24	78	167	46	27
Treated	26	51	19	37	139	21	15
Treated (and negative in rabbits)	11	26	0	0	49	0	0

TABLE IX

Summary of Experience with Eight Types of Pneumonia Treated with Homologous Antipneumococcus Rabbit Serum

Type	Number of cases	Age average	Multilobar consolidation	Bacteremia	Infected pleural exudate	Serum hours after onset	Serum c.c.	Crisis hours after serum	Mortality %
			Number	Number	Number	Average*	Average	Average	
I	25	33	9	11	2	77	134	19	4
II	10	40	3	1	2	68	225	41	10
V	1	2	1	0	1	13D	190	120	0
VI	2	1	2	2	2	7W	75	39	0
VII	6	48	1	1	0	55	160	17	0
VIII	7	40	2	2	0	35	160	19	0
XIV	2	39	0	0	0	66	270	54	0
XVIII	1	21	0	0	0	48	332	24	0
Average	..	35	..	..	..	...	165	27	..
Total	54	..	18	17	7	...	...	...	..
Per cent	..	..	33	31	13	...	...	...	3.7

* D indicates days. W indicates weeks.

the second and the fourth day after the onset of the disease. To these two cases, 360 and 180 c.c. of unconcentrated Type XIV rabbit antiserum were given. The acute signs of the disease disappeared sixty and forty-nine hours after the beginning of serum therapy. Both patients recovered. Serum sickness did not occur. In the Type XVIII case, serum therapy was begun forty-eight hours after the onset of the disease and 332 c.c. of unconcentrated serum were given intravenously. The acute signs of the disease disappeared twenty-four hours after the administration of serum. The patient developed serum sickness, but recovered.

DISCUSSION

In a discussion of the results obtained with type specific antipneumococcus rabbit serum as a therapeutic agent in lobar pneumonia, it seems best to consider separately a number of points.

Antipneumococcus rabbit serum, irrespective of type, frequently contains sufficient chill producing substances to cause severe reactions when injected intravenously in patients with lobar pneumonia. These reactions are not usually dangerous, but when extreme hyperpyrexia results as occurred in one instance in this series, severe symptoms may occur and death may follow despite active antifebrile therapy. It has been found[8] that the greater part of the chill producing substances can be removed from antipneumococcus rabbit serum by relatively simple procedures, that is by heating it to 56°C. for thirty minutes and subsequently absorbing it with sterile washed kaolin for fifteen hours at 4°C. It has also been found[3] that the extent of the mean thermal reaction which follows the intravenous injection of two c.c. of antipneumococcus rabbit serum in each of three normal rabbits, very closely parallels the severity of the chill reaction produced in human beings by the intravenous injection of the same serum. With this additional information it has been possible to restudy many of the various lots of antiserum which have been used therapeutically and to correlate the results previously obtained in human beings with those produced by the same sera when tested intravenously in normal rabbits. Sera which produced a mean thermal response of less than 1.2°F. after intravenous injection into three normal rabbits did not cause chill reactions when given intravenously to human

beings. Sera which caused a mean thermal response of more than 1.3°F. in rabbits did cause chill reactions in human beings and the severity of the chill was approximately proportional to the degree of thermal elevation produced in rabbits.

Forty different lots of antipneumococcus rabbit serum comprising the nine different types of antiserum have been used in these studies. A few patients have received two separate lots of homologous antiserum. Of the forty lots of antiserum, fourteen were entirely untreated, while twenty-six were treated in the manner described above. Of these latter treated lots, there were eleven which did not produce a significant thermal response after intravenous injection in normal rabbits. In Table VIII are shown in condensed form the experiences with untreated and with treated rabbit antiserum. It will be noted that the fourteen untreated lots of antiserum were injected in thirty-one patients, and that twenty-four of these patients had one or more chill reactions, an incidence of seventy-eight per cent. Of these fourteen untreated lots of antiserum, one hundred and sixty-seven injections were given to the thirty-one patients, and produced forty-six separate chill reactions, an incidence of twenty-seven per cent.

It will also be observed that the twenty-six lots of treated antiserum were injected in fifty-one patients, and that nineteen of these patients had one or more chill reactions, an incidence of thirty-seven per cent. Of these twenty-six treated lots, one hundred and thirty-nine injections were given to the fifty-one patients, and produced twenty-one separate chill reactions, an incidence of fifteen per cent. Eleven of the treated lots of antiserum were shown by intravenous test in rabbits to cause no significant thermal response. These eleven lots of antiserum were injected in twenty-six patients and did not cause a chill reaction. In all, forty-nine injections of these eleven lots were given without producing a single chill. These observations lead to the conclusion that the simple procedures previously mentioned definitely reduce the chill producing property of antipneumococcus rabbit serum. They also indicate the importance of testing the antiserum by intravenous injection in normal rabbits. At the present time no other method is known which will distinguish the potentially chill-producing rabbit antisera from those which will not cause such reactions.

Serum sickness is one of the unpleasant sequelae of serum therapy and although it is not serious it is capable of producing moderately disagreeable symptoms for a period of a few days. In a discussion of the occurrence of serum sickness after the administration of antipneumococcus rabbit serum, it must be borne in mind that unconcentrated rabbit antisera have been used throughout this study. It would therefore be illogical to make any comparative statements concerning the incidence of serum sickness after the administration of antipneumococcus sera from a species other than the rabbit, except when reference is made also to *unconcentrated* sera. In the last 100 cases of Type I pneumonia treated in the Hospital of The Rockefeller Institute with *unconcentrated* Type I antipneumococcus horse serum, serum sickness occurred in over ninety-five per cent of patients. MacKenzie and Hanger[4] have reported a serum sickness incidence of ninety per cent after the injection of similar horse antiserum in patients with lobar pneumonia. Of the fifty-nine patients who were treated with antipneumococcus rabbit serum and who recovered, twenty-four developed some evidence of serum sickness, an incidence of forty per cent. In sixteen cases the symptoms were mild, and in eight they were of moderate severity. In seventy-five per cent of cases in which serum sickness did develop it manifested itself almost entirely by cutaneous symptoms—urticaria, transient rashes, etc. The development of serum sickness was not related to the quantity of serum administered. In three patients more than 300 c.c. of antiserum were injected without the subsequent occurrence of serum sickness.

Prior to a discussion of the remainder of the points which have been raised by this study it will serve a useful purpose to separate the cases with Type III pneumonia from those with the other eight types of pneumonia, and to discuss the Type III cases first. As has been the case with antipneumococcus sera produced in other species, and as is still true even with rabbit antiserum, there is considerable difficulty in preparing highly potent antiserum against Type III pneumococcus. Certain factors underlying this difficulty are now fairly well understood.[5] Whether this can be entirely surmounted is a question which must be left to the future. It is apparent from a consideration of the results obtained in the first thirteen cases

of Type III pneumonia treated with Type III antipneumococcus rabbit serum, presented in Table III, that as yet no definite evidence exists that antiserum of this type has been of benefit in Type III pneumonia. In the discussion which follows, therefore, the cases of Type III pneumonia will be excluded from the consideration.

In Table IX are presented in summary the chief facts concerning fifty-four cases of lobar pneumonia of eight different types, which have been treated with unconcentrated homologous antipneumococcus rabbit serum.

It will be observed that consolidation of two or more lobes occurred in eighteen cases, or thirty-three per cent of the fifty-four cases treated. It has been adequately demonstrated by Cecil, Baldwin, and Larsen[10] that the mortality rate in non-serum treated cases is significantly increased as each additional lobe is consolidated. In this series of cases, all eighteen with multilobar consolidation who were treated with homologous antipneumococcus rabbit serum have recovered.

Pneumococcus bacteremia was present in seventeen cases, thirty-one per cent of the fifty-four cases treated. It has been well shown by Tilghman and Finland[6] and Bullowa and Wilcox[7] that in non-serum treated cases in which bacteremia occurs the mortality rate is markedly increased. In this series of cases, one patient with bacteremia (Type II pneumococcus) died, and sixteen patients recovered, a mortality rate of 5.9 per cent among the cases with pneumococcus bacteremia.

Pleural exudates infected with pneumococci were encountered in seven cases in this series of fifty-four, an incidence of thirteen per cent. Two of these seven, one with Type V pneumonia and another with Type VI, had frank empyemas before serum therapy was commenced. There remain five cases with infected exudates which had not progressed to the stage of empyema at the time serum therapy was begun. In three of these cases following the administration of antiserum, type specific antibody of rabbit origin became demonstrable in the exudates and subsequently the pneumococci disappeared. In two other cases with infected pleural exudates, antibody was not demonstrated in the exudates after the administration of rabbit antiserum, and empyema developed which necessitated surgical drainage. Finland[8] has

shown that horse antibody can penetrate the inflamed pleura but was unable to demonstrate homologous antibody when the pleural exudate was infected with pneumococci.

The rapidity with which recovery occurred after the administration of an adequate quantity of rabbit antiserum to patients with eight different types of lobar pneumonia has been one of the most striking findings in this study. In the fifty-four cases the average interval from the beginning of serum therapy until the disappearance of the acute signs of the disease was but twenty-seven hours. A total of eighteen patients received an adequate quantity of antiserum in a single injection, and in this group the interval from the beginning of serum therapy until the completion of crisis was only seven and one-half hours. Although there may not be any distinct advantage in very rapid recovery *per se*, nevertheless the fact that recovery can be brought about so rapidly as has been indicated, is additional evidence of the specificity and the effectiveness of the therapy employed.

It is apparent that the most important criterion of the success of any therapeutic measure in the treatment of a frequently fatal disease is the reduction in the mortality rate which can be attributed to the use of that agent. As a result of the numerous reports[9–13] of very large series of cases of lobar pneumonia not treated with antiserum, the mortality rates to be expected in many of the various types of the disease are clearly known. The increased mortality rates which occur in the presence of either multilobar consolidation, or pneumococcus bacteremia are also well-established. In this series of fifty-four cases of lobar pneumonia of eight different types, treated with unconcentrated homologous type specific antipneumococcus rabbit serum, despite the occurrence of multilobar consolidation in thirty-three per cent of cases, and pneumococcus bacteremia in thirty-one per cent of cases, there were but two deaths, a mortality rate of 3.7 per cent.

SUMMARY

1. Sixty-seven cases of lobar pneumonia, distributed among nine different types, have been treated with unconcentrated type specific antipneumococcus rabbit serum.

2. In thirteen cases of Type III pneumonia no evidence of the therapeutic effectiveness of Type III antipneumococcus rabbit serum was demonstrated.

3. In fifty-four cases of pneumonia due to eight other types of pneumococcus and treated with antipneumococcus rabbit serum of homologous type, the mortality rate was 3.7 per cent.

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THE EFFECT OF FORMALDEHYDE ON PNEUMOCOCCI

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Encapsulated pneumococci treated with sufficient concentrations of formaldehyde retain for some time their characteristic morphology, their positive reaction to the Gram stain, and their specific agglutinability in homologous antiserum. Moreover, rabbits and horses immunized by the intravenous route with formolized encapsulated pneumococci react with the production of the specific antibodies directed against the capsular polysaccharide of the bacterial cell used as antigen. It is generally considered, however, that pneumococcus antigens prepared by this technique are not very stable, and undergo with time a form of lysis accompanied by a loss of antigenicity (1).

It has been shown in a previous paper that the "capsular polysaccharide antigen" of Pneumococcus can be rendered ineffective by the action of an autolytic enzyme present in this bacterial species (2). Although this autolytic enzyme can be inactivated by a number of reagents, the inactivation is in many cases reversible (3–6). When, for instance, iodine is used in proper concentration to kill and "fix" pneumococci, the autolytic enzyme is inactivated and the cells retain their structure and their antigenicity; upon removal of the iodine, however, the enzyme may recover its activity and bring about lysis accompanied by loss of antigenicity (2, 5).

In the present study, an attempt has been made to analyze the action of formaldehyde on pneumococci in the light of the observations outlined above. Techniques to render stable the formolized pneumococcus antigens are also described.

EXPERIMENTAL

The bacteriological and immunological methods used in this study are the same as those described in a previous paper (2).

A commercial preparation of formalin (38 per cent formaldehyde) was used as source of formaldehyde.

The Effect of Different Concentrations of Formaldehyde on the Autolysis of Pneumococci.—A great many antiseptics have the apparently conflicting properties of activating the autolysis of different bacterial species when used in low concentrations, and of completely inhibiting

TABLE I

The Effect of Different Concentrations of Formaldehyde on the Viability and Autolysis of Pneumococci

Final concentration of formaldehyde	Growth on blood agar plates			Microscopic appearance of the cells	
	Formolized cultures incubated at 37°C. for				
	3 hrs.	24 hrs.	96 hrs.	24 hrs.	96 hrs.
per cent					
0.5	–	–	–	Gram-positive	Gram-positive
0.2	–	–	–	"	"
0.1	–	–	–	"	Mixture of Gram-positive and Gram-negative
0.05	–	–	–	Mixture of Gram-positive and Gram-negative	Gram-negative detritus
0.03	+	–	–	Gram-negative	" "
0.02	+	–	–	"	" "
0.01	+	+	–	Gram-positive	" "
0.005	+	+	–	"	" "
0	+	+	–	"	" "

+ indicates growth of pneumococci on blood agar plates.
– " no growth of " " " " "

the autolytic process when used in higher concentration (7, 8). The effect of different concentrations of formaldehyde on cultures of pneumococci is considered in Experiment 1.

Experiment 1.—A plain broth culture of Type III pneumococci 8 hours old was distributed in 5 cc. amounts into test tubes; the culture had reached a final pH of 7.1. Varying amounts of formaldehyde were added to the different samples to give final concentrations ranging from 0.005 to 0.5 per cent, and the formolized cultures were incubated at 37°C. and examined after different intervals of time. The presence of living cells was determined by streaking the cultures on blood agar plates, and the degree of autolysis was followed by microscopic examination of films stained by the Gram technique. The results are presented in Table I.

The results of Experiment 1 show that an amount of formaldehyde corresponding to a final concentration of 0.05 per cent was sufficient to sterilize a culture of Type III pneumococci in 3 hours at 37°C. This amount, however, was not capable of preventing autolysis. In fact the cells treated with the smaller concentrations of formaldehyde (0.02 to 0.05 per cent) underwent autolysis more rapidly than the control cells. The cells treated with the largest concentrations of the antiseptic (0.2 per cent and 0.5 per cent) remained well formed and Gram-positive; in other words they were fixed by the reagent.

Partial Lysis of Formolized Pneumococci Washed Free of Formaldehyde.—The cells in Experiment 1 had remained in the presence of formaldehyde throughout the period of observation; the behavior of pneumococci killed with an amount of formaldehyde sufficient to inhibit the autolytic enzyme, then washed free of the antiseptic, is considered in Experiment 2.

Experiment 2.—500 cc. of plain broth culture of Type III pneumococcus was treated with enough formaldehyde to give a final concentration of 0.5 per cent. The formolized culture was kept at room temperature for 24 hours; the cells were then separated by centrifugalization, washed twice, and resuspended in 50 cc. physiological saline solution. The bacterial suspension consisting of well formed Gram-positive cells was then divided into two equal fractions, one of which received immediately 0.5 per cent formaldehyde. Both fractions were kept at room temperature and the microscopic appearance of the cells followed by the Gram stain.

The cells maintained in the presence of formaldehyde remained well formed and Gram-positive throughout the period of observation (4 weeks). Some of the cells in the suspension washed free of the antiseptic had on the contrary become Gram-negative within 24 hours, and hardly any Gram-positive cocci could be seen after 72 hours. The change from a Gram-positive to a Gram-negative state was accompanied by a reduction in size of the cocci, but no real disintegration of the cells could be observed. No further evidence of lysis appeared on prolonged incubation. The experiment was repeated at ice box temperature (5°C.) and at 37°C. The results were identical with the only difference that the rate of change was slower at 5°C. and faster at 37°C. It is therefore apparent that whereas pneumococci treated with an excess of formaldehyde retain their morphological

and staining characteristics as long as they are kept in the presence of this agent, they may undergo a limited form of lysis when washed free of the antiseptic.

Factors Affecting the Lysis of Formolized Pneumococci.—It has been shown elsewhere that the inactivation of some of the pneumococcus enzymes by iodine is a reversible process; these enzymes recover their activity when the iodine is removed by reducing agents (5, 6). It appeared possible that the lysis suffered by formolized pneumococci washed free of formaldehyde was due to the reactivation of some autolytic enzyme. To test this hypothesis, formolized pneumococci were kept under a variety of experimental conditions in order to control the action of the autolytic enzymes.

Experiment 3.—20 cc. of formalin were added to 1500 cc. of a young culture of Type III Pneumococcus. The formolized culture was kept at room temperature for 24 hours, then divided into six equal fractions and centrifugalized. The formolized cells were suspended in 5 cc. amounts of the following media.

(*a*) M/20 phosphate buffer pH 7.0
(*b*) " " " " " + 0.1 cc. formalin
(*c*) " " " " " ; this suspension was immediately heated at 75°C. for 20 min.
(*d*) " K_2HPO_4 (final reaction pH 8.0)
(*e*) M/20 acetic acid (final reaction pH 4.2)
(*f*) Lugol iodine solution

These cell suspensions were kept at 37°C. and stained by the Gram technique after different intervals of time.

After 24 hours, the cells in suspension (*a*) (neutral reaction, unheated and in the absence of additional formaldehyde or iodine) had become Gram-negative. In all the other preparations, the cells remained Gram-positive throughout the period of observation (2 weeks at 37°C.).

In other words, the change from a Gram-positive to a Gram-negative state is inhibited by the presence of formaldehyde or iodine, by heating the cell suspension, or by maintaining it at an acid or alkaline reaction. These findings may be interpreted as follows. The change from Gram-positive to Gram-negative is caused by an enzyme which is inactive in the presence of formaldehyde (*b*) or iodine (*f*) but which recovers its activity when the formaldehyde is removed and the prepa-

ration is incubated at neutral reaction (*a*); the enzyme is irreversibly inactivated by heating at 75°C. (*c*) and does not function at acid (*e*) or alkaline (*d*) reaction. To substantiate this hypothesis it was of interest to study the effect of the autolytic enzyme of Pneumococcus on formolized cells of this bacterial species.

The Effect of the Autolytic Enzyme of Pneumococcus on Formolized Pneumococci.—It is known that the autolytic enzyme of Pneumococcus can be obtained in an active form from autolysates and extracts of pneumococci (9, 10, 5). The effect of this enzyme preparation on formolized pneumococci is described in the following experiment.

Experiment 4.—3 cc. of formalin were added to 300 cc. of a young culture of Pneumococcus Type III in plain broth. The formolized culture was allowed to stand for 24 hours at room temperature. The cells were then separated by centrifugalization, washed once in saline, and divided into two equal fractions. One fraction was resuspended in 15 cc. M/20 K_2HPO_4 (the final pH of the suspension was 7.8); the other was resuspended in 15 cc. M/20 phosphate buffer at pH 7.0 and this suspension immediately heated at 75°C. for 20 minutes. Both suspensions consisted of well formed Gram-positive cells.

The bacteriolytic enzyme was prepared according to a technique previously described (5). The cells from 150 cc. of a broth culture of R Pneumococcus, derived from Type II, were resuspended in 15 cc. of distilled water and allowed to autolyze in the presence of toluol for 48 hours at 37°C. The Gram-negative detritus was then removed by centrifugalization and the active supernate used as enzyme.

To 1.0 cc. amounts of the two formolized bacterial suspensions (heated at pH 7.0, or resuspended in M/20 K_2HPO_4 and unheated) was added 1.0 cc. to 0.1 cc. of the solution of bacteriolytic enzyme. The mixtures were made up to 2.0 cc. volume with saline and incubated at 37°C. for 24 hours. At the end of this time films of the different mixtures were stained by the Gram technique to estimate the action of the enzyme on the two bacterial suspensions.

It appears from the results of Experiment 4 that the bacteriolytic enzyme of pneumococcus is capable of attacking pneumococci killed with formaldehyde. The enzymatic digestion however does not bring about a disintegration, a real "lysis" of the cell bodies; on the contrary the cells become Gram-negative, but retain their general morphology. In this respect, formolized cells differ from heat-killed cells which are completely disintegrated by sufficient amounts of the bacteriolytic enzyme. It is of special interest that the enzyme renders the formolized cells Gram-negative in a medium at pH 7.8,

i.e. under conditions where the formaldehyde is not likely to dissociate from its combination with the cell structure. Since formolized pneumococci kept at pH 7.8 do not of themselves become Gram-negative, whereas they can be attacked by an active preparation of the enzyme, it seems logical to assume that fixation by formaldehyde is due not to its action on the cell substrate, but to the inactivation of the bacteriolytic enzyme. The results of Experiment 3 indicate that under proper conditions, the fixation of the cell, and therefore the inactivation of the enzyme, are reversible phenomena.

The Effect of Formaldehyde upon the Type Specific Antigenicity of Encapsulated Pneumococci.—Fresh preparations of formolized encapsulated pneumococci (Gram-positive) are capable of inciting the production of the type specific carbohydrate antibodies when injected into rabbits by the intravenous route. It has been shown elsewhere however, that the capsular polysaccharide antigen of pneumococci is inactivated by the action of an autolytic enzyme obtained from the same bacterial species (2). It appeared therefore possible that the partial lysis suffered by formolized pneumococci washed free of the antiseptic (Experiments 2 and 3) would be associated with a loss of type specific antigenicity. This is illustrated in the following experiment.

TABLE II

The Effect of the Bacteriolytic Enzyme of Pneumococcus on Formolized Pneumococci

Amount of enzyme	Appearance of cells at end of incubation period	
	Formolized cells at pH 7.8	Formolized cells heated at pH 7.0
cc.		
1.0	Gram-negative cocci	Gram-negative cocci
0.1	" "	" "
0	Gram-positive cocci	Gram-positive cocci

Experiment 5.—The same cell suspensions described in Experiment 3 were used for the immunization of rabbits by the intravenous route. The immunizing dose was the equivalent of 2 cc. of culture daily; three rabbits were used for each preparation. The animals were bled 1 week after the completion of the second course of immunization; their sera were tested for the presence of type specific agglutinins and for precipitins for the homologous capsular polysaccharide (Type III).

The sera of the three animals immunized with the formolized cells which had become Gram-negative (preparation (*a*)) failed to show any type specific agglutinin or precipitin in any dilution. On the contrary the sera of all the animals which had received the formolized cells maintained Gram-positive by destruction or inactivation of the autolytic enzyme exhibited high agglutinating titers and also precipitated the capsular polysaccharide of Type III Pneumococcus. Although there were, of course, marked individual variations between the animals, the best titers were obtained with preparations (*c*) (formolized cells heated at 75°C.) and (*d*) (formolized cells resuspended in dibasic potassium phosphate). Several other experiments were therefore instituted to confirm the antigenic efficacy of these two bacterial suspensions. Twenty-four rabbits were immunized with formolized-heated pneumococci Type III and 18 with formolized cells resuspended in M/20 dibasic potassium phosphate; the amounts of antigen used were the same as in Experiment 5. The sera of the forty-two animals showed type specific agglutinins in titers ranging from 1:40 to 1:640; the prozone phenomenon was observed with the sera of the highest titers. All but four sera were capable of precipitating the capsular polysaccharide of Type III Pneumococcus; the precipitin reaction was very intense in the best sera and was still detectable in a dilution of serum of 1/20. No appreciable difference could be detected between the two antigen preparations.

DISCUSSION

Formaldehyde, when added in sufficiently large concentration to cultures of pneumococci, acts as a fixing agent and preserves the Gram-positive character and the morphological integrity of the cells. When used in lower concentrations on the contrary, it activates markedly the autolytic disintegration of the cells of the same bacterial species. A similar phenomenon has been observed with different types of antiseptics acting on different microorganisms, namely that the same agent may activate, or on the contrary completely stop, the autolytic process according to the concentration in which it is used (7, 8). It has been suggested elsewhere (8) that large concentrations of the antiseptic (formaldehyde in this case) inactivate the autolytic enzymes, whereas smaller concentrations, by interfering with the normal physi-

ology of the cell, cause the autolytic enzymes to act on their specific cellular substrate.

When the cells fixed with sufficient concentrations of formaldehyde are washed free of the antiseptic and resuspended in a physiological medium at neutral reaction, they undergo a partial lysis. The lysis is not complete however, but leaves the cells in the form of small Gram-negative cocci; this "first phase of autolysis" had already been recognized by the use of other techniques in an earlier study (5).

What is the nature of the reaction which renders the formolized cells Gram-negative? One might assume that formaldehyde combines in a reversible manner with the substance or structure responsible for the Gram stain, and that the Gram-positive property is lost when the formaldehyde is allowed to dissociate from its complex with the cell. The following facts are in agreement with this hypothesis. Formaldehyde dissociates from its combinations more readily at pH 7.0 than at pH 8.0, and it has been shown in Experiment 3 that formolized cells may become Gram-negative at pH 7.0 but remain Gram-positive at pH 8.0. On the other hand however, formolized cells resuspended in acetic acid (pH 4.4) also remain Gram-positive although the formaldehyde complexes are least stable at acid reactions. It appears therefore that mere dissociation of formaldehyde from the Gram-positive structure cannot account for the change in staining reaction. The following theory seems to provide a satisfactory explanation of the facts described in Experiment 3.

Pneumococci are known to contain an enzyme capable of destroying the Gram-positive structure of the cells of this bacterial species (9, 10, 5). This enzyme is inactivated by a number of reagents, for example, iodine and formaldehyde. It has been shown that iodine inactivation is reversible (3, 4). We may suppose that formaldehyde inactivation also reverses when the bacterial cells are washed free of the antiseptic at neutral reaction; under these conditions, the enzyme recovers its activity and renders the cells Gram-negative. When, however, the formolized cells are rapidly heated at 75°C., the enzymes are destroyed and no autolysis can take place even at pH 7.0.

Both acid (pH 4.2) and alkaline (pH 7.8) reactions inhibit the action of the enzyme but through an entirely different mechanism. In the acid medium, the enzyme is freed from its combination with formalde-

hyde; but pH 4.2 is outside the range of enzymatic activity and therefore no lysis can take place. On the contrary it has been shown in Experiment 4 that an active preparation of the enzyme can attack formolized cells at pH 7.8. Since formolized pneumococci do not undergo any autolytic change when kept at this reaction, we are led to assume that the enzyme is maintained in an inactive form in the alkaline medium.

On the basis of these observations it is possible to prepare stable suspensions of formolized pneumococci by the following method. Enough formaldehyde is added to the culture to give a final concentration of 0.5 per cent; this stops immediately all autolytic process. The cells can then be separated at leisure from the medium and resuspended under such conditions that no further autolytic action can take place, for instance in a solution of M/20 dibasic phosphate or in an acetate buffer at acid reaction (pH 4.2), or in any physiological solution in which they are immediately heated at 75°C. for 20 minutes.

Cells of encapsulated pneumococci treated by any of these methods have proven to function as very effective type specific antigens even when used in small amounts; they incite rabbits to produce high titers of the type specific antibodies directed against the capsular polysaccharide of the bacterial cells. The results obtained with Type III Pneumococcus are the more striking when one considers that cells of this particular type are notoriously poor antigens. On the contrary, formolized pneumococci which have been allowed to become Gram-negative by removal of the antiseptic and incubation at neutral reaction, entirely fail to incite the production of the type specific carbohydrate antibodies in rabbits immunized by the intravenous route. This finding once more emphasizes the close correlation between the particular antigen concerned, and the structure responsible for the Gram-positive character of the cell (2).

The experimental results described in this paper deal with the effect of formaldehyde on Type III Pneumococcus. Identical results, however, have been obtained with pneumococci of Types I and II, and the methods of preparation of pneumococcus antigens outlined above have been used successfully for the production in rabbits of therapeutic antisera for the different pneumococcus types (11).

SUMMARY

When used in low concentration, formaldehyde increases the rate of autolytic disintegration of pneumococci whereas in large concentrations it completely inhibits autolysis and preserves both the morphological and staining characteristics of the cells.

Pneumococci treated with large concentrations of formaldehyde, then washed free of the antiseptic and resuspended in physiological solutions, rapidly undergo a change which renders them Gram-negative and smaller. The lysis is only partial, however, and is not accompanied by an actual disintegration of the cell. It is caused by the autolytic enzyme of the cell which remains inactive in the presence of an excess of formaldehyde but recovers its activity when the cells are resuspended in a neutral medium after removal of the antiseptic. If the autolytic enzyme is irreversibly inactivated by heating, or maintained inactive in acid or alkaline reaction, the formolized cells retain their staining characteristics and morphological integrity.

Formolized pneumococci which have become Gram-negative owing to the action of their autolytic enzyme, fail to elicit the type specific carbohydrate antibodies in rabbits. Formolized pneumococci in which the autolytic enzyme has been destroyed or maintained inactive, and which have retained their Gram-positive character, function as a very effective type specific antigen in the rabbit.

These observations emphasize once more the close relation between the Gram-positive structure of pneumococci and the capsular polysaccharide antigen of the cell. They can be used as a basis for the preparation of suspensions of formolized pneumococci which are stable and very effective as type specific antigens.

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THE ACTION OF SULFANILAMIDE IN RHEUMATIC FEVER

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The hypothesis that hemolytic streptococci have an etiologic rôle in many cases of rheumatic fever makes it highly desirable to determine the action of sulfanilamide in this disease. In undertaking this study, however, we understood that it might not be possible to evaluate its action with the same criteria that have been applied in using it in the treatment of such diseases as streptococcic bacteremia, meningitis or erysipelas, for if streptococci are indeed the causative agents in rheumatic fever they seem to act in a more complicated manner than as simple infectious agents. The present study therefore has theoretical as well as practical implications.

The literature has few references to this subject. Klee and Römer[1] observed a patient with acute polyarthritis and endocarditis who was treated with massive doses of prontosil and "cured" in eight weeks. They also observed some patients with acute polyarthritis who did not respond to salicylate and aminopyrine therapy but improved when given the disodium salt of 4-sulfamidophenyl-2′-azo-7′-acetylamino-1′-hydroxynaphthalene-3′, 6′-disulfonic acid (prontosil); but no favorable effect of this drug was noted in chronic arthritis, even of a secondary nature. Massell[2] found no beneficial effect from prontosil in two patients with frank rheumatic fever or in two others with chorea. Six rheumatic subjects who contracted hemolytic streptococcus infections were given this drug in an attempt to prevent recurrence of rheumatic fever, but the disease developed in two and one died. The same number of relapses occurred in six control pa-

1. Klee, P., and Römer, H.: Prontosil bei Streptokokkenerkrankungen, Deutsche med. Wchnschr. **61**: 253 (Feb. 15) 1935.

2. Massell, B. F.: Studies on the Use of Prontylin in Rheumatic Fever, New England J. Med. **216**: 487 (March 18) 1937.

tients, who were not treated with the drug. Peters and Havard[3] compared the complications in 150 patients with scarlet fever treated with parabenzylamino-benzene-sulfonamide with those in a like number of patients not so treated (fifty of the latter had serum). Endocarditis developed in three of the first group, compared with nine of the second; in three of the first and ten of the second group rheumatism developed. There is no report of the action of this drug after the development of rheumatic symptoms.

The low toxicity of prontosil and sulfanilamide for laboratory animals has not been altogether paralleled in man, and there is a growing list of cases of severe and even serious drug intoxication. Most patients have some degree of gastric disturbance, running the gamut from "indigestion"[4] to anorexia, nausea and vomiting. Three fourths of a large series of patients observed by Long and Bliss[4] had cyanosis. In some instances this is due to sulfhemoglobinemia[5] and in others to methemoglobinemia,[4] but often its cause is undetermined.[6] All patients taking therapeutic doses of the drug probably have some degree of acidosis, although it is not usually severe enough to be detected clinically.[7] Both prontosil and sulfanilamide have definite fever-inducing capacities,[4] and both at times have a toxic action on the blood or blood-forming organs. Massell[2] and Plumer[8] both

3. Peters, B. A., and Havard, R. V.: Chemotherapy of Streptococcal Infections with p-Benzylamino-Benzene-Sulphonamide, Lancet 1: 1273 (May 29) 1937.

4. Long, P. H., and Bliss, Eleanor A.: The Use of Para Amino Benzene-Sulphonamide (Sulphanilamide) or Its Derivatives in the Treatment of Infections Due to Beta Hemolytic Streptococci, Pneumococci and Meningococci, South. M. J. 30: 479 (May) 1937.

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6. Marshall, E. K., Jr., and Walzl, E. M.: On the Cyanosis from Sulfanilamide, Bull. Johns Hopkins Hosp. 61: 140 (Aug.) 1937.

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8. Plumer, H. E.: Correspondence, New England J. Med. 216: 711 (April 22) 1937.

observed cases of leukopenia, and Harvey and Janeway[9] carefully studied three cases in which acute hemolytic anemia was apparently induced by these drugs. They stated that the hepatic function is depressed, as shown by the bromsulfalein test. Most authors agree that the kidney is relatively little if at all affected, but in one of our patients, to be described, suppression of urine, then albuminuria and cylindruria followed severe intoxication with sulfanilamide.

While the severe infections for which the drug was first employed may have been accessory factors in the intoxication, the warning of Reuter[10] concerning the probable harmful action of the drug has been amply justified in the number of severe toxic symptoms that have recently been recorded in patients with such mild diseases as simple gonococcic urethritis.[11]

Method

Because rheumatic fever varies widely in duration, intensity and clinical course, the evaluation of therapeutic measures is extremely difficult, especially if they appear to be beneficial; hence in the present

9. Harvey, A. M., and Janeway, C. A.: The Development of Acute Hemolytic Anemia During the Administration of Sulfanilamide (Para-Aminobenzene-sulfonamide), J. A. M. A. 109: 12 (July 3) 1937.

10. Reuter, F. A.: The Use of Sulfanilamide in the Treatment of Gonorrhea: Report of Results in One Hundred Cases. M. Ann. District of Columbia 6: 117 (May) 1937.

11. Among the toxic symptoms recorded are those reported in THE JOURNAL for Sept. 25, 1937:

Kohn, S. E.: Acute Hemolytic Anemia During Treatment with Sulfanilamide, p. 1005.

Bucy, P. C.: Toxic Optic Neuritis Resulting from Sulfanilamide, p. 1007.

Menville, J. G., and Archinard, J. J.: Skin Eruptions in Patients Receiving Sulfanilamide, p. 1008.

Goodman, M. H., and Levy, C. S.: The Development of a Cutaneous Eruption (Toxicodermatosis) During the Administration of Sulfanilamide: Report of Two Cases, p. 1009.

Frank, L. J.: Dermatitis from Sulfanilamide, p. 1011.

Schonberg, I. L.: Purpuric and Scarlatiniform Eruption Following Sulfanilamide, p. 1035.

Newman, B. A., and Sharlit, Herman: Sulfanilamide: A Photosensitizing Agent of the Skin, p. 1036.

Salvin, Monte: Hypersensitivity to Sulfanilamide, p. 1038.

study we selected only patients from whom a definite answer was expected. Patients improving with simple rest and also those immediately requiring the well established therapeutic measures were eliminated; for example, patients with severe pancarditis. In all instances therefore several days' observation was needed to make a rough estimate of the probable course of the case under investigation. Patients observed previously were especially useful, for when two or more former attacks had followed a definite pattern it seemed probable that the present illness would resemble the former ones. This occurred in some of the cases included in the present series.

The daily charting of all possible clinical manifestations, together with the therapeutic agents, is helpful, because the effect of the drug under investigation can readily be compared with that of other therapeutic measures; this is especially useful in the case of rheumatic fever, for which there are several drugs with definite and apparently beneficial effects on some of the most distressing manifestations of the disease. The precautions and measures outlined were closely adhered to during these investigations. An abstract of the course of events is given in the following report of cases, together with as much of the data as it was practical to chart.

REPORT OF CASES

CASE 1.—*History.*—P. H., a woman, aged 21, admitted Dec. 18, 1936, on the ninth day of an indeterminate number of recurrences of a chronic type of rheumatic fever, when 7 years of age had the first definite symptoms of heart disease and when 10 the first definite polyarthritis. She had been treated six years and two years previously in this hospital for a chronic type of rheumatic polyarthritis and carditis. Mitral and aortic valves were both involved. The arthritis had usually been characterized by pain and tenderness, with a minimum of swelling and redness.

Course.—Slowly increasing pain and tenderness had been present in many joints for eight days, with precordial pain for the past two days. On admission there were precordial pain and tenderness, mitral systolic, aortic systolic and diastolic murmurs, and pain and tenderness in the right shoulder, hips, knees and feet. The blood pressure was 124 systolic, 40 diastolic. During the following eight days there was an even, low grade fever, but a rising pulse rate, continuous precordial pain and tenderness, additional painful and tender joints and a rising erythrocyte sedimentation rate all indicated a progressive rheumatic infection.

On the seventeenth day the administration of sulfanilamide was started, 2 Gm. being given for two days and then 3 Gm., a dosage of 70 mg. per kilogram of body

weight, for four days. The only beneficial effect noted was some temporary diminution in precordial pain and tenderness. On the other hand, a slight rise in temperature and a marked rise in the pulse rate took place, and additional joints became painful and tender. On the seventh day of this therapy, nausea appeared, cyanosis became distinct, precordial pain was more marked and a further increase in the erythrocyte sedimentation rate was noted. Examination of the blood showed no sulfhemoglobin but some methemoglobin. The administration of sulfanilamide was stopped and aminopyrine was given, with disappearance of the toxic symptoms and slow diminution in the signs of rheumatic activity.

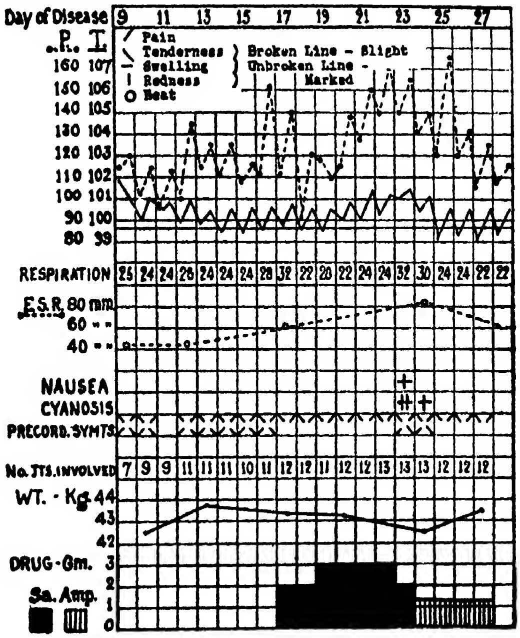

CHART 1.—Course in case 1. In the charts *Sa.* indicates sulfanilamide, *Amp.* aminopyrine and *Asp.* acetylsalicylic acid. Symbols used are explained at the top of this chart.

That the rheumatic infection was not stopped was shown by continuation of the abnormal erythrocyte sedimentation rate, tachycardia and low grade fever.

No hemolytic streptococci were found in cultures of material from the nose and throat, and the antistreptolysin titer, which was 150 units at the time of admission, never rose any higher. In this patient, therefore, there was no direct evidence of recent hemolytic streptococcus infection.

Summary.—Seven days' treatment with fair sized doses of sulfanilamide apparently did not favorably affect the rheumatic process in a patient with a low grade, subacute type of infection. On the other hand, general toxic symptoms, methemoglobinemia and a marked rise in the pulse rate gave definite indications

that the drug had an unfavorable action, and these signs disappeared when its administration was stopped and aminopyrine was given.

CASE 2.—*History.*—H. K., a man, aged 24, was admitted Feb. 15, 1937, on the twenty-fifth day of the fifth definite attack of rheumatic fever. All of the attacks had been characterized by chronicity, marked and persistent erythema marginatum and subcutaneous nodules. In the first attack, at the age of 7, there were, in addition, chorea, carditis and pleurisy. In subsequent relapses the fever had been rather low grade and the arthritis mild but persistent. There had been a progressive increase of cardiac involvement, so that the aortic valve was very incompetent; there was also a loud mitral systolic murmur.

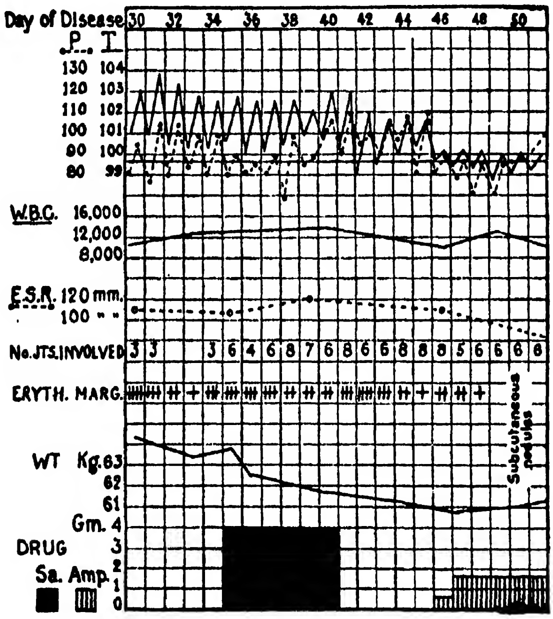

CHART 2.—Course in case 2.

Course.—Beginning on January 22, there was a three day attack of "grip." A period of latency of thirteen days followed, and then pain appeared in the calf muscles, followed two days later by mild arthritis in the knees and ankles, which spread to the elbows and shoulders and was accompanied by a fever as high as 103 F. On admission the cardiac murmurs were the same as had been noted previously; the blood pressure was 130 systolic, 50 diastolic. The arthritis was limited to the left arm, and there was beginning erythema marginatum. During the next ten days the fever ranged mostly between 100 and 102.5 F., but the pulse rate was relatively slow. The erythema marginatum became intense, but the arthritic lesions were relatively mild. The erythrocyte sedimentation rate continued at about 112 mm. per hour, and low grade leukocytosis persisted.

As the course resembled so closely the pattern previously observed, the case seemed ideal for a trial of sulfanilamide; so, on the thirty-fifth day, the administration of this drug was started, and it was continued for six days in doses of 4 Gm. (62 mg. per kilogram of body weight). There was a definite though moderate increase in both fever and pulse rate, together with a "dopey feeling," but, with the exception of a fall in weight, the toxic symptoms shown by the other patients were lacking. The rheumatic manifestations continued, with an increase in the number of joints involved and many new cutaneous lesions. When administration of the drug was stopped, there was a fall in temperature and the patient felt better. After a drug-free interval of five days, aminopyrine was given, with a prompt fall in the temperature and pulse rate and marked amelioration of most of the symptoms. On the fiftieth day, however, a crop of subcutaneous nodules appeared, and new ones were observed for the following two weeks. Thus the picture previously observed was completed, and the patient was not discharged until 140 days after the onset of his illness.

Although no hemolytic streptococci were recovered from this patient, the antistreptolysin titer in the serum rose from 300 units at the time of admission to 500 units ten days later and 600 units by the fiftieth day, when the subcutaneous nodules appeared. The concentration of these antibodies remained at this high level for about two months, then fell slowly. It thus seems probable that hemolytic streptococci had recently been active but that sulfanilamide had no effect on the continued production of antistreptolysin.

Summary.—In a patient who had shown a remarkable constant type of rheumatic fever during four previous attacks, with erythema marginatum and subcutaneous nodules as prominent features, sulfanilamide, in what would be considered adequate dosage, had no beneficial effect on the usual course of events. On the other hand, it seemed to have a pyrogenic and pulse-accelerating influence, in spite of the absence of most of the other usual signs of sulfanilamide intoxication.

CASE 3.—*History.*—C. C., a girl, aged 10, was admitted Dec. 3, 1936, on the twenty-third day of her second attack of rheumatic fever. At the age of 3 she had acute arthritis, involving both legs, for two weeks; no mention was made of cardiac disease at that time.

Course.—November 11 the patient had sore throat and headache lasting one day. She was well for six days, and on the eighth day arthritis of the knees appeared, followed by migratory polyarthritis in both legs and arms. Two days before admission, the twenty-first day of the disease, she received her first medical attention; she then had fever and polyarthritis, which were relieved by some medicine. On admission the temperature was 101 F. and the pulse rate 120. A mitral systolic murmur was present, and the arthritis was limited to the joints of the right arm and hand. During the next two days the temperature and the pulse rate were high and numerous new joints were involved; hence she was given sulfanilamide, first in doses of 1.2 Gm. daily for two days; i.e., 50 mg. per kilogram of body weight. The fever and pulse rate became higher, and precordial pain and tenderness and a pericardial rub appeared. The dose was increased to 2 Gm.

(80 mg. per kilogram of body weight), and, while the arthritis diminished in some joints, others were involved. Precordial pain and tenderness were more marked, and the pericardial rub was heard over a larger area. The pulse rate rose to 150 and the respiratory rate to 40. There were almost complete anorexia, with nausea and vomiting, and an increase in leukocytosis.

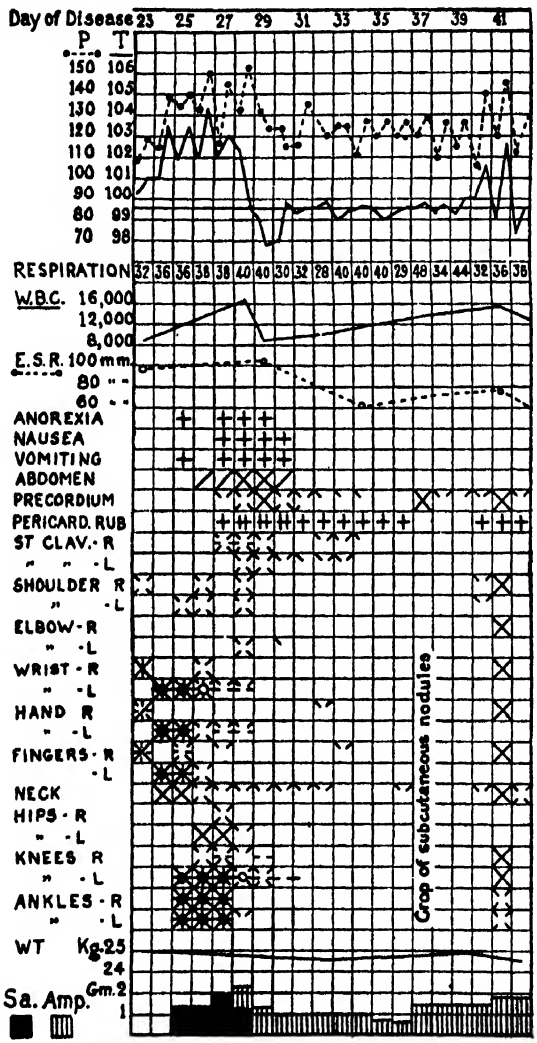

CHART 3.—Course in case 3.

By the twenty-eighth day (the fourth day of sulfanilamide medication) the condition was such that aminopyrine seemed urgently indicated. One gram of this drug induced a marked fall in temperature and relief of the polyarthritis, but the anorexia, nausea and vomiting continued through the following day. The precordial and abdominal pains were slower in responding, and the rapid

pulse and respiratory rates continued, probably because of the pericarditis, for roentgenograms revealed distinct cardiac dilatation.

Twelve days after the beginning of sulfanilamide therapy, a crop of subacute rheumatic nodules appeared, and new ones were noted for the next week; macroscopic nodules persisted for a month.

On the eighty-fourth day, moderate fever, polyarthritis, cardiac dilatation and pleurisy reappeared, but they were relieved promptly by increasing the dose of aminopyrine from 1.2 to 1.8 Gm. Tonsillectomy was performed on the one hundred and sixteenth day. The patient was discharged on the hundred and forty-second day. The heart was distinctly larger than on admission, no new murmurs were present but a musical mitral systolic murmur continued, and there were probably some pericardial adhesions.

No hemolytic streptococci were recovered from cultures of material from the throat made shortly after admission or from the tonsils removed on the one hundred and sixteenth day. The antistreptolysin titer in the serum obtained on the twenty-third day was 700 units, where it remained during the next two months; it then slowly decreased but was still 300 units six months after the onset of the disease. This is presumptive evidence of recent hemolytic streptococcus infection.

Summary.—Although receiving sulfanilamide in relatively large doses and to the limit of tolerance, the patient continued to show increasing acute signs of arthritis and pancarditis. Furthermore, the subsequent appearance of subcutaneous rheumatic nodules proved that in this patient the drug did not inhibit the development of the proliferative manifestations of the rheumatic infection.

CASE 4.—*History.*—S. J., a boy, aged 13, admitted May 20, 1937, on the eleventh day of the fourth attack of rheumatic fever, had his first attack of the disease at the age of 9 and two or three relapses subsequently.

Course.—May 10 the patient had a cold in the head and marked coryza. On the eighth day there were fever and arthritis, which began in the right knee and left ankle. Two days later, while still at home, the patient received 0.66 Gm. of sulfanilamide, and he received 0.33 Gm. the day of admission. On admission, May 20, the temperature was 104 F., the pulse rate 120, and the respiratory rate 38; there were arthritis of the right knee, gallop rhythm and mitral systolic and aortic diastolic murmurs. Two days later the arthritis had regressed from the right knee but had migrated to the left knee and the toes of the left foot. The fever continued. As the two day control period indicated a spreading rheumatic process, the administration of sulfanilamide was started in doses of 4 Gm. a day, the equivalent of 54 mg. per kilogram of body weight. The general course is indicated on chart 4. While the arthritis, present at the beginning of medication, disappeared, the fever and rapid pulse rate continued; the respirations became very rapid, cyanosis increased and anorexia became marked. Arthritis of the right wrist appeared and increased. In fact, the general aspect of the patient indicated need for definite antirheumatic medication, so aminopyrine was given, with the usual spectacular improvement, which continued uninterrupted. The

loss in weight could be attributed both to an antiobesity diet and to almost complete anorexia.

That the patient was satisfactorily absorbing sulfanilamide was proved by the results of blood analysis on the third day of this therapy, when the total concentration of sulfanilamide was 7.4 mg. per hundred cubic centimeters and the concentration of the nonconjugated form 5.7 mg.[12] Two days later the total was 8.25 mg., of which 6.9 mg. was nonconjugated, and thirty-six hours after administration of the drug was stopped the total concentration was 1.67 mg., of which 0.9 mg.

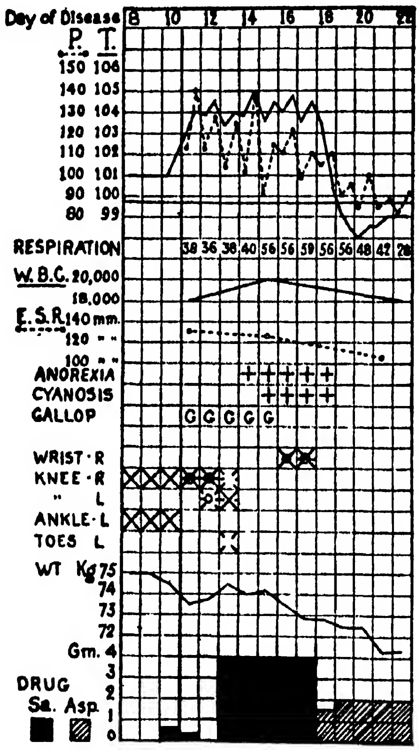

CHART 4.—Course in case 4.

was nonconjugated. Thus the conjugated sulfanilamide on the three dates was 23, 16 and 46 per cent, respectively.

The antistreptolysin concentration in the blood serum, which was 1,600 units on admission, dropped to 1,000 and then went to 1,200 units, where it remained for over two months.

Summary.—In the fourth definite attack of rheumatic polyarthritis, a boy of

12. Sulfanilamide estimations were made according to the method of E. K. Marshall Jr. (Determination of Sulfanilamide in Blood and Urine, Proc. Soc. Exper. Biol. & Med. 36: 422 [April] 1937).

13, with probable involvement of the mitral valve and definite involvement of the aortic valve, received 4 Gm. of sulfanilamide daily for five days, a total of 20 Gm., which produced a concentration of at least 8 mg. per hundred cubic centimeters in the blood. While arthritis, present at the beginning, disappeared, new joints became involved, and a continued high fever and pulse rate, a mounting respiratory rate, cyanosis, nausea and increasing toxicity, with continuing marked leukocytosis, all pointed to little beneficial effect compared with what would have been expected early in an acute streptococcic infection. The contrast with the rapid antisymptomatic influence of aminopyrine was most striking.

CASE 5.—*History.*—F. P., a man, aged 20, was admitted May 24, 1937, on the fifth day of an acute attack of rheumatic fever. This was at least the sixth recurrence of the disease in a patient in whom, at the age of 11, the first manifestation was chorea. He had been treated in this hospital at various times for chorea, polyarthritis and carditis, and during this period both mitral and aortic valvular disease had developed.

Course.—No symptoms of preliminary infection of the upper respiratory tract were present. Four days prior to admission the patient had severe epistaxis, weakness, fever, migratory polyarthritis and persistent precordial pain. These symptoms were much relieved by self medication with 2 Gm. daily doses of aminopyrine, taken for the past two days. On admission the temperature was 101 F. and the pulse rate 100. The only complaint was pain and tenderness of the right knee. Apical systolic and aortic diastolic murmurs were present. The blood pressure was 135 systolic, 50 diastolic. During the next four days the temperature rose to 103 F. but the pulse remained relatively slow. On the eighth day anorexia and vomiting were present on two occasions. On the ninth day, because of recurring polyarthritis, the administration of sulfanilamide was started; 4 Gm. daily, i.e., 50 mg. per kilogram of body weight, was given. Cyanosis appeared and the anorexia continued. On the tenth day, with the same dose, the fever was higher, the pulse rate more rapid and the cyanosis more marked, and there were complete anorexia and more arthritis. On the following day the patient appeared much sicker, and, although fewer joints were involved, the right wrist was more markedly inflamed and the fingers of the left hand were painful and tender. Dyspnea and cyanosis were marked. The appearance of gallop rhythm indicated more marked cardiac involvement. After the third day of sulfanilamide the patient's condition became such that the administration of this drug was stopped and aminopyrine was given. Within a few hours there was symptomatic relief, and the following day the picture was entirely changed, although some cyanosis and dyspnea continued for two days.

That the rheumatic infection was not stopped by this therapy was shown by the development of pleurisy, together with clinical and electrocardiographic signs of active myocarditis; also, the erythrocyte sedimentation rate was rising and the weight falling during the succeeding month.

While the maximal dose of sulfanilamide was only 50 mg. per kilogram of body weight daily, the concentration in the blood on the third day of medication was 7.5

mg. per hundred cubic centimeters, with 5.9 mg. nonconjugated. Two days after administration of the drug was discontinued there was still a total of 1.5 mg., with 1 mg. nonconjugated. The blood taken at the time of the most marked cyanosis showed no sulfhemoglobin.[13] No hemolytic streptococci were recovered from cultures of material from the nose and throat. The antistreptolysin titer in the blood serum was slightly more than 150 units at the time of admission and

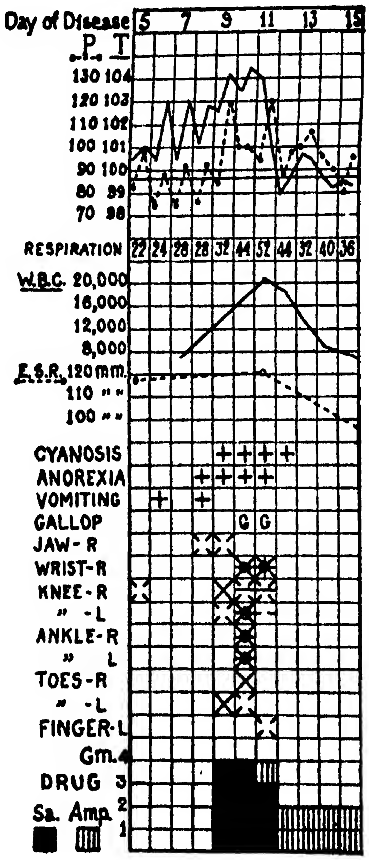

CHART 5.—Course in case 5.

rose ten days later to 250 units, where it remained two weeks, then fell slowly. This is presumptive evidence of recent hemolytic streptococcus infection.

Summary.—No antirheumatic influence was exerted by sulfanilamide when given in moderate doses to a patient during the second week of a severe relapse. Even this moderate dosage induced toxic symptoms so severe that discontinuance of the medication was indicated on the third day. The contrast with the effect

13. Dr. G. I. Lavin performed these tests.

of aminopyrine, exhibited on two occasions, was most striking. The severe cyanosis was not due to sulfhemoglobinemia.

CASE 6.—*History.*—G. M., a girl, aged 7, admitted May 15, 1937, on the fifteenth day of the first attack of rheumatic fever, had a history of numerous infections of the upper respiratory tract, the last one of which began fifteen days previous to admission. The patient had precordial pain on the third, fourth and fifth days and pain and tenderness of the elbow and knees from the fifth day until admission. She was given three tablets a day of acetylsalicylic acid from the eighth to the thirteenth day with slight improvement of the arthritis. A culture

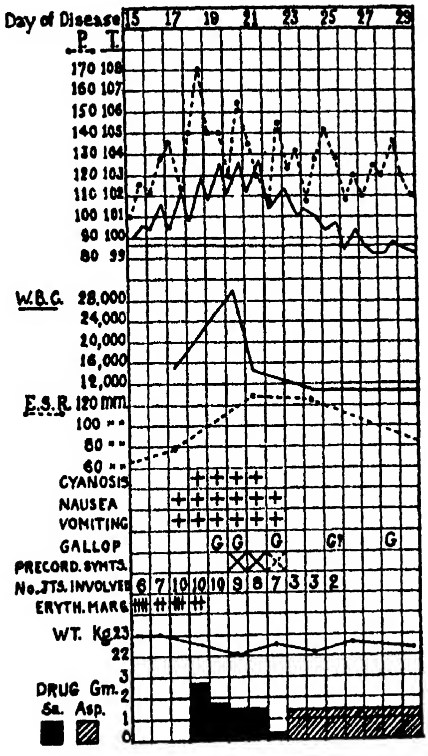

CHART 6.—Course in case 6.

of material from the throat on the twelfth day showed 10 per cent matt colonies of hemolytic streptococci. Erythema marginatum appeared on the fourteenth day.

Course.—The patient when admitted had a temperature of 100 F., a pulse rate of 100, extensive erythema marginatum, and pain and tenderness, but no swelling or redness of the shoulders, elbows and knees. A blowing apical systolic murmur was present. About 5 per cent of the colonies in a culture of material from the throat were hemolytic streptococci. During the next three days there were mounting fever and pulse rate, spreading arthritis, diminishing erythema and a

rising erythrocyte sedimentation rate. Although hemolytic streptococci persisted in cultures of material from the throat, the colonies were fewer.

Because of increasing symptoms, sulfanilamide therapy was instituted on the eighteenth day; 2.6 Gm. was given, i.e., 113 mg. per kilogram of body weight, in order to obtain rapid saturation. Cyanosis appeared by evening; previously existing nausea and vomiting continued. The next day the dose was dropped to 1.6 Gm. and the two following days to 1.3 Gm. each day. The fever increased, and the pulse rate became very rapid; polyarthritis, consisting of pain and tenderness, continued, and precordial pain and tenderness appeared. Cyanosis, nausea and vomiting became more marked, leukocytosis continued and the erythrocyte sedimentation rate rose from 80 to 130 mm. per hour.

Because of the absence of beneficial effect, all medication was stopped on the twenty-second day, and acetylsalicylic acid therapy was started the twenty-third day, with steady improvement. The systolic murmur assumed a musical character, which persisted until about the forty-eighth day, when it diminished in intensity, but a loud aortic diastolic murmur appeared and has persisted.

Hemolytic streptococci were not found in cultures of material from the throat after the second day of sulfanilamide treatment. The total concentration of sulfanilamide in the blood serum on the evening of the second day of drug therapy was 6 mg. per hundred cubic centimeters, with 5.15 mg. nonconjugated. Two days after the administration of this drug was discontinued there was still a total of 1 mg. of sulfanilamide per hundred cubic centimeters, with 0.83 mg. nonconjugated.

Summary.—There was little diminution in the arthritis, but an increase in the cardiac symptoms, leukocytosis and erythrocyte sedimentation rate, together with increasing toxicity, in a child with a first attack of rheumatic fever who was given all the sulfanilamide she could retain by mouth and who evidently absorbed most of it into the blood stream. The only beneficial effect noted was disappearance of erythema marginatum. That the cardiac involvement was not arrested was shown by the appearance of definite aortic regurgitation one month later. The effect on the streptococcic flora in the throat was indeterminate, because the number of colonies was diminishing before sulfanilamide was given.

Case 7.—*History.*—C. K., a boy, aged 15, admitted May 10, 1937, on the ninth day of a definite recurrence of rheumatism, had had many attacks of sore throat earlier in life but fewer in the past four years. At the age of 7 a tonsillectomy was performed, and three months later the first joint pains were noted. He spent six months in another hospital, where he was told he had heart disease. He was at home eight months, out of bed a few weeks and then in the hospital five and a half months. He was well for nine months, then in bed almost continuously for nine months. In the spring of 1936 he was again treated for arthritis, in another hospital for three months and at home for two months. Each attack of acute arthritis was relieved with aminopyrine. He stated that in the past seven years "there was never a week without joint pains" but that in the past eight months he had been much better, especially during the last four months. He had

been told repeatedly that he had heart disease, but he had had no symptoms of congestive failure.

Course.—May 2 the patient had a sore throat, with slight fever; he then had no symptoms until May 7, when fever appeared, the cervical lymph nodes were tender and swollen and there was definite arthritis of the knees, with pains in the upper extremities. Self medication with 2 Gm. of aminopyrine on the seventh and eighth days gave almost complete relief from arthritic pain, but the swelling in the left knee continued.

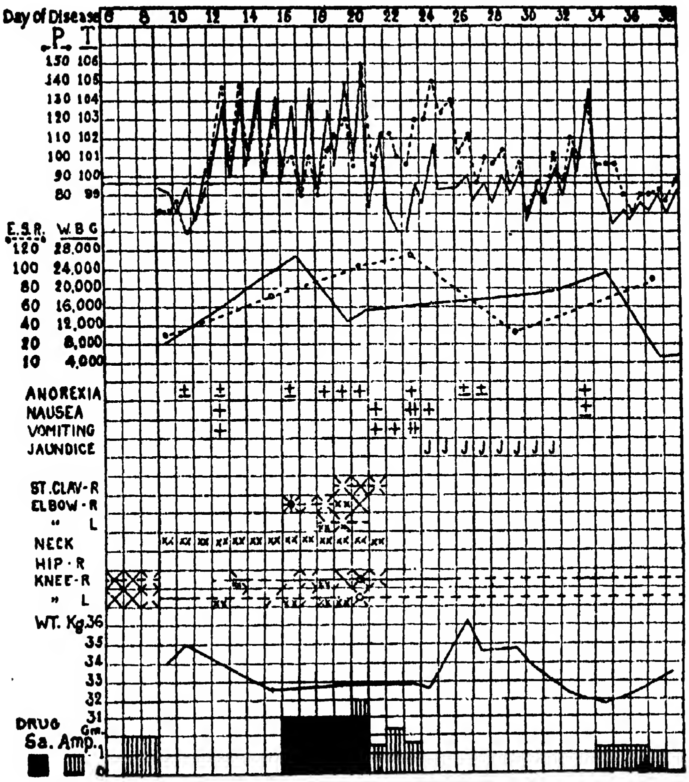

CHART 7.—Course in case 7.

On admission the temperature and the pulse were normal; the patient did not look acutely ill, although he had a sallow color. The heart was normal in size, with only a slight apical systolic murmur. The spleen was moderately enlarged. The neck was stiff, and a roentgenogram showed fusion of the laminae of the third, fourth and fifth cervical vertebrae. The fingers were slightly spindle shaped, with overextension of the first interphalangeal joint and flexion of the distal interphalangeal joints. Marked effusion was present in the left knee. As the influence of the aminopyrine wore off, there appeared a septic fever, rapid pulse rate and recurring arthritis of the knees, characterized by marked effusion but rela-

tively little pain and tenderness. An increase in the leukocytosis and the erythrocyte sedimentation rate and migratory polyarthritis gave evidence by the sixteenth day of progressive rheumatic infection. Whether the condition was true rheumatic fever or an acute phase of chronic rheumatoid arthritis was not definite, but some features favored the latter.

On the sixteenth day the administration of sulfanilamide was started, 3 Gm. daily, or 94 mg. per kilogram of body weight, being given. This dose was given for five days with no beneficial effects; on the contrary, the clinical picture showed a rising pulse rate, a still higher erythrocyte sedimentation rate, persisting leukocytosis and involvement of new joints without any disappearance of the inflammation in those previously involved. Because of the discomfort and the history of beneficial effects of aminopyrine, 1 Gm. of this drug was given on the twentieth day, with the usual favorable influence on the arthritis and the temperature. This drug was given three days more, but during this period toxic symptoms, noted at the end of the sulfanilamide therapy, increased, so that finally the

Sulfanilamide Content of the Blood in Case 7

Day of Disease	Day of Sulfanilamide	Total, Mg. per 100 Cc.	Nonconjugated, Mg. per 100 Cc.	Conjugated, Mg. per 100 Cc.	Conjugated, Percentage
18th	3d	8.3*	6.5	1.8	22
20th	5th (stopped)	8.2	5.5	2.7	32
23d	.....	3.8*	1.1	2.7	71
24th	.....	3.3	1.1	2.2	66
26th	.....	1.0	0.3	0.7	70
27th	.....	0.3	0.2	0.1	33

* Serum analyzed.

patient was constantly nauseated and unable to retain any food or fluids given by mouth. There was also complete suppression of urine for forty-eight hours. Fluids were supplied by hypodermoclysis, intravenous infusion of dextrose and proctoclysis, and this treatment was accompanied by diminution in nausea and vomiting, with renewed ability to take nourishment by mouth. On the twenty-fourth day of the disease a generalized morbiliform rash appeared for only a few hours; icterus was noted, which became more marked, then slowly diminished and finally disappeared after being present a week.

On the twenty-fourth day the patient appeared very anemic, and a blood count showed 2,400,000 erythrocytes and 51 per cent hemoglobin. The icteric index was 25, with the serum bilirubin 2.5 per cent. An extremely high content of blood pigment and the presence of urobilin in the urine suggested some hepatic damage. Then edema appeared in the face and legs, and there was a rapid increase of over 3 Kg., about 10 per cent, in body weight. As the jaundice disappeared the fever recurred, with an increase of effusion in the right knee; hence aminopyrine was again given, with the customary clinical improvement. This time, however, the

exhibition of this drug was followed by a drop in leukocytes to 4,600, with only 30 per cent of granulocytes, so it was discontinued, with a fairly rapid return of the leukocytes toward normal. Clinical improvement continued. The anemia diminished, and the only untoward sign that appeared was hematuria, first macroscopic and then microscopic. The urea clearance on the forty-eighth day was normal, and urine concentration tests showed normal ability to concentrate. The results of several analyses of whole blood or serum for the content of sulfanilamide are shown in the accompanying table.

While the patient was taking the drug in as large doses as he could tolerate, the concentration in the blood was over 8 mg. per hundred cubic centimeters. More interesting still was the retention of unusually large amounts for several days after it was no longer given, and during this period the conjugated form was about 70 per cent. The anuria and the subsequent low renal capacity doubtless caused the slow excretion.

Although no hemolytic streptococci were recovered from this patient, the antistreptolysin titer indicated that these microorganisms were probably active. On admission the titer in the serum was only 50 units; six days later, just before the administration of sulfanilamide was started, it was 700 units; two weeks later it had risen to 3,000 units, and it remained at 2,400 units for over two months. Obviously, in this patient the drug did not inhibit the formation of large amounts of this antibody.

Summary.—Although the type of rheumatism could not be definitely determined, it was acute and followed a definite severe angina. The high antistreptolysin curve indicated a recent hemolytic streptococcus infection. Five days of intensive sulfanilamide therapy was accompanied by an increase in the arthritis and induced severe intoxication, with renal and hepatic damage, and anemia, with acute hemolytic jaundice, from which the patient recovered. The contrast between the lack of therapeutic effect and the marked drug intoxication was especially noteworthy.

CASE 8.—*History.*—J. R., a boy, aged 13, admitted March 16, 1937, a week after the onset of his present illness, was said to have had rheumatism annually since 1931. On admission there were signs of consolidation in the left lower lobe, slight cardiac enlargement, mitral systolic and aortic diastolic murmurs, pains in the knees and shoulders, erythema marginatum, albuminuria and hematuria. A few colonies of group A hemolytic streptococci were recovered from cultures of material from the throat.

Course.—During the succeeding two months there were alternating periods of activity and a tendency toward improvement. May 29 a tonsillectomy and adenoidectomy were performed. Group B hemolytic streptococci were isolated from the tonsils. After the operation there was a flare-up in the rheumatic activity. During August a granulomatous area in the nasopharynx was found, and from it group A hemolytic streptococci were repeatedly cultured. There was also evidence of ethmoiditis. With the patient taking daily doses of 1.8 Gm. of acetylsalicylic acid the temperature ranged between 98 and 100 F. and the pulse rate

between 80 and 110. September 24, the two hundredth day of the present attack, 2 Gm. of sulfanilamide was given and the following day 3.3 Gm. An elevation of temperature to 102.6 F. followed, with a pulse rate of 148, marked nausea and anorexia and a remarkable increase in the erythema marginatum. Because these symptoms of toxicity persisted on the third day of medication, only 0.6 Gm. was given. Toxic symptoms persisted for twenty-four hours, then disappeared. This episode was followed by disappearance of the hemolytic streptococci from the nasal cultures, disappearance of the erythema marginatum and some improvement for about three weeks. Then another cycle of rheumatic activity appeared.

Summary.—Six grams of sulfanilamide given during three days to a boy with chronic rheumatic fever induced fever, tachycardia, nausea and anorexia, marked general depression and a remarkable increase in erythema marginatum. That these effects were due to the action of the drug was proved by their disappearance when it was withdrawn.

COMMENT

Although only a few cases were studied, if each is considered as a single valid test there are enough to establish the fact that, under the conditions of these observations, sulfanilamide has little, if any, detectable beneficial effect on the course of rheumatic fever, once the condition is well established. Practically all its common manifestations except chorea were represented in this group, and none responded favorably. On the other hand, the toxic effects of the drug were especially marked in these patients, and as a result certain rheumatic manifestations seemed to be intensified.

The criticism might be advanced that the ineffectiveness of sulfanilamide here recorded was due either to insufficient daily dosage or to relatively short periods of administration. In answer it must be noted that all patients except patient 2 received the drug until gastric symptoms made it unpractical to administer more. In five there was marked and in two others moderate cyanosis, and in most there was a distinct increase in respiratory rate, which might be attributed either directly to the drug or to an intensification of the disease.

Other toxic symptoms apparently directly attributable to the drug were increasing fever and pulse rate. Thrice these decreased shortly after administration of the drug was stopped, and a fever of 102.6 F. in case 8 the day following the first doses of sulfanilamide left little doubt that the drug was a definite pyrogenic factor. In other cases

urgent symptoms indicated the use of aminopyrine; therefore, the fever-inducing rôle of sulfanilamide could not be so well determined. It seems highly probable, however, that the drug helped to increase the fever in all instances. Moreover, Long and Bliss[14] observed that fever was the most common toxic symptom induced by sulfanilamide. The time relationship between the beginning of drug treatment and the onset of fever differed from the delayed reaction reported by Hageman and Blake;[15] in our cases the onset was immediate. This feature alone might be sufficient contraindication for using sulfanilamide in the presence of rheumatic fever, for hyperpyrexia is a very serious manifestation of this disease; it is necessary to guard constantly against any factor that might induce an elevation of temperature, because this symptom of itself could determine a fatal outcome.

The accelerated pulse rate might have been due to one or more of several influences: (*a*) a direct toxic action of the drug on the heart, (*b*) acidosis and the condition of the blood that was manifest as cyanosis, (*c*) added fever, though in several instances the acceleration of the pulse was out of proportion to the increased temperature, and (*d*) an actual increase in the rheumatic infection. In any event, a markedly accelerated heart rate is a distinctly undesirable influence in any therapeutic measure applied to rheumatic fever, for with this acceleration there is a proportional increase in the functional trauma to which the cardiac valves and the myocardium are subjected while in an inflamed condition, and, even though the outcome is eventual recovery, the amount of residual scarring is probably increased.

Most drugs have toxic capacities that must be considered in relationship to their therapeutic effects, and if the latter outweigh the former the drugs are considered satisfactory. This seems to be the case with the sulfanilamide treatment of serious acute hemolytic streptococcus infections as well as of infections induced by meningococci[16] and gonococci.[10] It is therefore necessary to investigate

14. Long, P. H., and Bliss, Eleanor A.: Para-Aminobenzenesulfonamide and Its Derivatives, Arch. Surg. **34:** 351 (Feb.) 1937.

15. Hageman, P. O., and Blake, F. G.: A Specific Febrile Reaction to Sulfanilamide, J. A. M. A. **109:** 642 (Aug. 28) 1937.

16. Schwentker, F. F.; Gelman, Sidney, and Long, P. H.: The Treatment of Meningococcic Meningitis with Sulfanilamide, J. A. M. A. **108:** 1407 (April 24) 1937.

carefully the influence of this drug on all the manifestations of rheumatic fever that can be closely observed. The difficulty of accurately estimating its influence on the febrile course has been discussed. Its effect on arthritis appears more easily determinable, even though some of the signs are entirely subjective, as was the case in three of our patients in whom pain and tenderness persisted and possibly became more troublesome. In four other patients, however, there were definite objective signs of migration to formerly symptomless joints, even though some joints improved that were previously involved. In other words, the arthritis behaved much as would have been expected had the patients received no treatment.

Erythema marginatum is one of the characteristic signs of continuing rheumatic intoxication. In one patient (patient 6) it disappeared even though other signs of the disease persisted; in a second (patient 2), in whom erythema marginatum had always been an outstanding feature, many new lesions appeared daily during the six days of sulfanilamide therapy, and in a third (patient 8) it was much aggravated. In patient 2 subcutaneous nodules also developed within a fortnight of the time he was fully under the influence of this drug, and the same phenomenon was observed in patient 3. Sulfanilamide therefore exerted no appreciable therapeutic effect on the immediate acute exudative manifestations or any prophylactic influence on the more remote proliferative features that could be accurately observed.

While the effect on the visceral lesions was more difficult to determine, pericarditis developed in patient 3 while she was taking sulfanilamide, and in several there was evidence of myocardial involvement either while they were taking the drug or shortly thereafter. This evidence, in conjunction with the well known tendency of rheumatic myocarditis to develop simultaneously with subcutaneous nodules, makes it highly probable that the cardiac manifestations were not favorably influenced by sulfanilamide.

The leukocyte curve and the erythrocyte sedimentation rate are considered useful objective criteria of the course of rheumatic infection. There was an increase in leukocytosis in all seven patients in whom this feature was accurately measured; in five the count rose to 20,000 or more, and in the two others to 13,000 and 16,000, respectively.

Sulfanilamide, likewise, seemed to exert no favorable influence on the erythrocyte sedimentation rate. In one patient a measurement of 125 mm. per hour remained practically the same; in another the rate increased from 115 to 120 mm., and in the others the rise from previous abnormal levels was 10, 15, 20, 30 and 50 mm., respectively. In only one did a falling curve continue downward. While a fall in so short a period might not have been expected or considered clear evidence of therapeutic failure of the drug, the almost consistent increase in rate pointed definitely to such a failure.

Theoretically, the question might be raised as to whether the lack of beneficial effect of sulfanilamide in these patients indicated that the hemolytic streptococcus had no influence in causing their rheumatic state. On the other hand, it is possible that, once this state is established, the drug will have no antirheumatic action, even though it may eliminate the streptococci. It is therefore essential to present the evidence that hemolytic streptococcus infection was present in these subjects, both before and after treatment. This evidence was the isolation of such cocci and the presence of antihemolysins—so-called antistreptolysins—in the blood serum.

In only two patients were hemolytic streptococci recovered from cultures of material from the nose and throat. In explanation may be advanced the argument that no patient was seen at the time of onset of the upper respiratory infection, which was definitely present in six of the eight, and that hemolytic streptococci, which might have been present at the onset, could easily have disappeared from the throat in the interval.

The antistreptolysin[17] curves for six of the eight patients were indicative of fairly recent infection with these micro-organisms. The two other curves, showing titers of only 100 to 150 units, might be considered as equivocal evidence; however, the repeated isolation of hemolytic streptococci from the nasopharynx of one of the patients pointed to a focal infection with these bacteria. Of the six with significant antibody titers, two (5 and 6) had curves quickly rising to 250 units from an earlier level of 150, and in one of these the titer

17. Todd, E. W.: Antihaemolysin Titres in Haemolytic Streptococcal Infections and Their Significance in Rheumatic Fever, Brit. J. Exper. Path. 13: 248 (June) 1932.

eventually reached 350. Patient 2 in the fourth week had a titer of 350 units, which later doubled in strength. A fifth, patient 3, maintained a level of 700 units from the fourth week to the third month. Patient 4 showed 1,600 units in the second week, from 1,000 to 1,200 units during the next four weeks and a slowly falling curve thereafter. For the final patient (7) the curve rose rapidly from 50 units on the ninth day to 700 on the fifteenth and to about 3,000 on the twenty-ninth and then varied between this level and 2,500 for many weeks. This was the patient who suffered the severe sulfanilamide intoxication.

Sulfanilamide therapy had no uniform effect on the antistreptolysin curve. The relation of the titers with respect to exhibition of the drug was as follows: In three patients the titer remained level at 100, 150 and 700 units, respectively; in one it remained level at 500 units and two weeks later rose to 600; in one it rose from about 175 to 250 units and in another from 150 to 350; in patient 7 it rose from 700 to 2,400 units during treatment with the drug and a week later to almost 3,000 units. In only one patient (4) was there a fall during sulfanilamide therapy; the titer dropped from 1,600 to 1,000 units and subsequently rose to 1,200. As the range in curves is about what might have been expected in an untreated group of patients with rheumatic fever of comparable severity, there is little evidence that the formation of antistreptolysin was influenced in any way by the drug. On the other hand, there was strong presumptive evidence that at least six of the patients were responding normally with formation of antibody to recent infection with hemolytic streptococci.

That significant amounts of this antibody should have developed prior to their receiving sulfanilamide is not surprising, because the stimulus to the formation of antistreptolysin doubtless was active from two to four weeks before the therapy was instituted. However, the prolonged, and in some cases rising, antibody curves following the use of such an antistreptococcic agent is of theoretical interest for the following reasons: It seems probable that the course of the antistreptolysin curve might give some indication of the possible elimination—or lack of elimination—of the streptococci as a result of the sulfanilamide therapy, for Todd[18] has found that the titer in

18. Todd, E. W.: Personal communication to the authors.

the serum of animals falls rapidly as soon as the injection of the antigen is discontinued, and Coburn[19] recorded that guinea pigs maintain high titers when carrying foci of infection. The lack of a definite fall in the antibody curve within a few weeks of sulfanilamide treatment is therefore presumptive evidence of continued production of streptolysin. This evidence is in line with our observation of several other patients, suffering from simple tonsillitis, in whose serum fairly high antistreptolysin titers developed in spite of their receiving sulfanilamide early in the course of the infection.

Knowledge of the mechanism whereby streptococci may induce the manifestations of rheumatic fever in many, if not all, instances is distinctly theoretical. The hypothesis guiding our general investigations may be formulated as follows: The primary hemolytic streptococcus infection is followed quickly by a state of antitoxic immunity, in contrast to a relatively delayed type-specific antibacterial immunity,[20] and a state of bacterial hypersensitivity (hyperergy to streptococci) is concomitantly induced. With the development of partial immunity, the persisting streptococci are reduced to a state of relative avirulence for the individual but continue to be active in the tonsils, lymph nodes, sinuses and other tissues, where they set up focal infections, which are ideal sites for the further, continuous stimulation of a hypersensitive state of the entire body. Moreover, the persistence of streptococci in these foci results in the elaboration of poisonous substances, either from the bacterial bodies or from the patient's tissues, or from both, which irritate and damage certain portions of the hypersensitive mesenchymal system. Assuming that this mechanism accounts, in part, for the damage inflicted on the rheumatic patient's tissues, it is possible that the injury could be stopped either by lowering the hypersensitivity of the tissues or by removing completely the streptococci from the foci.

That the hypersensitive state of some of the tissues was not lowered is shown by the persistence of marked cutaneous reactivity to intra-

19. (*a*) Coburn, A. F.: Personal communication to the authors; (*b*) Observations on the Mechanism of Rheumatic Fever, Lancet **2:** 1025 (Oct. 31) 1936.

20. Swift, H. F., and Hodge, B. E.: Type-Specific Anti-M Precipitins in Rheumatic and Non-Rheumatic Patients with Hemolytic Streptococcal Infections, Proc. Soc. Exper. Biol. & Med. **34:** 849 (June) 1936. Coburn.[19b]

dermal injection of hemolytic streptococcus extract in several of the patients. Moreover, in studying the action of sulfanilamide in guinea pigs infected with hemolytic streptococci, we[21] found that neither in vivo nor in vitro was it possible to demonstrate any distinct diminution in the hypersensitivity of the tissues of animals taking large doses of the drug.

It seems therefore that the only effect which might be expected from this antistreptococcus agent in a rheumatic patient infected with hemolytic streptococci is a diminution in the amount of poisonous substances that might be flowing out of infected foci because most of the bacteria acting in them had been killed. Colebrook and his co-workers,[22] however, have observed that prontosil-treated patients who have recovered from puerperal fever continue to discharge streptococci in the lochia for long periods. We have grown hemolytic streptococci from material from the throats of some sulfanilamide-treated patients for several weeks after they had recovered from tonsillitis, and Longcope[23] has recovered similar micro-organisms from excised tonsils of patients who had received large amounts of the drug. Several observers have reported the persistence of latent foci of hemolytic streptococci in mice treated with large doses of prontosil or sulfanilamide.[24] With such evidence available, it would be surprising if all streptococci were eliminated from the deep-seated foci in our rheumatic patients, who could tolerate only relatively moderate amounts of sulfanilamide; hence it seems highly probable that bacterial toxic substances continued to be elaborated and thrown off from infected foci.

For the foregoing reasons it is obvious that the therapeutic results in this series of patients do not shed much new light on the possible relationship of hemolytic streptococcus infections to rheumatic fever. Nevertheless, these investigations clearly demonstrate that sulfanil-

21. Swift, H. F., and Moen, J. K.: Unpublished observation.

22. Colebrook, Leonard; Kenny, Méave, and others: Treatment of Human Puerperal Infections, and of Experimental Infections in Mice, with Prontosil, Lancet 1: 1279 (June 6) 1936.

23. Longcope, W. T.: Personal communication to the authors.

24. Long, P. H., and Bliss, E. A.: Para-Amino-Benzene-Sulfonamide and Its Derivatives, J. A. M. A. 108: 32 (Jan. 2) 1937. Colebrook and his associates.[22]

amide, when given in as large doses as can be tolerated after the onset of rheumatic fever, exercises little, if any, ameliorating influence on the course of the rheumatism. Moreover, because the toxic by-effects seem to be specially marked in patients with this infection, the drug may have a deleterious influence on the course of the disease. The prophylactic action of sulfanilamide in rheumatic subjects infected with hemolytic streptococci is still to be determined.

CONCLUSION

The toxic action of sulfanilamide in active rheumatic fever so far outweighs the beneficial therapeutic effect that its administration to patients with this disease does not seem justified.

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CYTOLOGIC STUDIES ON RHEUMATIC FEVER

III. A COMPARISON OF CELLS OF SUBCUTANEOUS NODULES FROM PATIENTS WITH RHEUMATIC FEVER, RHEUMATOID ARTHRITIS AND SYPHILIS

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Although the subcutaneous nodules of rheumatic fever have received relatively greater attention than those of rheumatoid arthritis, the latter have been studied by a considerable number of investigators.[1] The clinical similarity between the nodules in the two diseases was early recognized and led to the suggestion[1a] that rheumatic fever and rheumatoid arthritis might be merely different manifestations of a single disease. More recently it has been shown[2] that the two types of lesions are even more strikingly similar in their histologic structure. An extensive and quite separate literature has accumulated, also, concerning the clinically very similar subcutaneous (juxto-articular) nodules encountered among patients with syphilis and those with yaws.[3]

1. (*a*) Futcher, T. B.: Bull. Johns Hopkins Hosp. 6:133, 1895. (*b*) Hawthorne, C. O.: Rheumatism, Rheumatoid Arthritis and Subcutaneous Nodules, London, J. & A. Churchill, 1900. (*c*) Wick, L.: Wien. med. Presse 45:1117, 1173, 1234, 1280 and 1324, 1904; Wien. med. Wchnschr. 31:1804, 1910. (*d*) Garrod, A. E., in Allbutt, T. C., and Rolleston, H. D.: A System of Medicine, New York, The Macmillan Company, 1910, vol. 3, p. 3. (*e*) Crouzon, O., and Bertrand, I.: Bull. et mém. Soc. méd. d. hôp. de Paris 50:1401, 1926. (*f*) Dawson, M. H., and Boots, R. H.: J. A. M. A. 95:1894, 1930.

2. (*a*) Coates, V., and Coombs, C. F.: Arch. Dis. Childhood 1:183, 1928. (*b*) Freund, E.: Wien. Arch. f. inn. Med. 16:73, 1928. (*c*) Coates, V.: Proc. Roy. Soc. Med. 23:587, 1930. (*d*) Clawson, B. J., and Wetherby, M.: Am. J. Path. 8:283, 1932. (*e*) Klinge, F., and Grzimek, N.: Virchows Arch. f. path. Anat. 284:646, 1932. (*f*) Dawson, M. H.: J. Exper. Med. 57:845, 1933. Dawson and Boots.[1f]

3. (*a*) Mayer, M., and Nauck, E. G.: Nodositas juxta-articularis, in Jadas-

In an earlier paper[4] a study of supravitally stained scrapings of rheumatic subcutaneous nodules was reported. The appearance of the characteristic cells of these lesions when stained with neutral red and Janus green was described, and it was concluded: (*a*) that these cells do not show with neutral red the reactions which distinguish monocytes, epithelioid cells and clasmatocytes, (*b*) that they probably arise from the undifferentiated mesenchymal cells of loose connective tissue and (*c*) that these observations probably apply also to the characteristic cells of rheumatic granulomas in the heart. Since the cardiac lesions do not lend themselves to study by supravital staining, the last conclusion was drawn purely from inference and was based on the clinical and pathologic evidence that rheumatic subcutaneous nodules are essentially the same as rheumatic granulomas elsewhere in the body. Some doubt has been cast on the validity of this assumption, however, by Hopkins' statement[3d] that the syphilitic nodules studied by him could not be differentiated microscopically from those associated with arthritis. This suggested the possibility that subcutaneous nodules might be merely nonspecific lesions occurring in various diseases and not representative of the characteristic pathologic changes of those diseases as found in other parts of the body. To test this possibility the present study was undertaken with the purpose of comparing supravitally stained preparations of subcutaneous nodules from patients with rheumatic fever, rheumatoid arthritis and syphilis.

MATERIAL AND TECHNIC

The tissues studied consisted of eleven rheumatic subcutaneous nodules previously described,[4] eight nodules from patients with rheumatoid arthritis,[5] two syphilitic juxto-articular nodules,[6] one gouty

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sohn, J.: Handbuch der Haut- und Geschlechtskrankheiten, Berlin, Julius Springer, 1932. (*b*) Hoffman, H.: Juxtaarticulare Knoten, in Jadassohn, J.: Handbuch der Haut- und Geschlechtskrankheiten, Berlin, Julius Springer, 1932. (*c*) Brunsting, L. A.: Am. J. Syph. **15**:42, 1931. (*d*) Hopkins, H. H.: Bull. Johns Hopkins Hosp. **49**:5, 1931.

4. McEwen, C.: J. Exper Med. **55**:745, 1932.

5. Five of the patients had been referred for biopsy and study from the clinic for patients with arthritis of the Presbyterian Hospital, New York, by Dr. R. H. Boots and Dr. M. H. Dawson.

6. The opportunity to study one of these patients was given me by Dr. J. P.

tophus and several sebaceous cysts, as well as normal human and rabbit subcutaneous tissues.

The rheumatic nodules were obtained from eleven children from 6 to 14 years of age, all of whom had typical rheumatic fever with characteristic cardiac involvement. None had clinical or serologic evidence of syphilis. The excised nodules had been present approximately from ten to ninety days prior to biopsy and measured from 0.25 to 0.5 cm. in diameter.

Each of the eight patients with rheumatoid arthritis had chronic deforming polyarthritis typical of that disease. The youngest patient was 30 and the oldest 62 years of age. Four were females, and four, males. None had signs of heart disease, and none gave a history of having had rheumatic fever. There was no reason to suspect syphilis in any, and all gave negative Wassermann reactions. The earliest nodule in point of development had been present three weeks, and the oldest, two years. In size the lesions varied from 1 by 1 by 0.25 to 4 by 2 by 1 cm.; each excised lesion had been located at the characteristic site on the extensor surface of the forearm, just distal to the olecranon.

Both patients with syphilitic nodules were males. One was white and the other a Negro. Both were in the tertiary stage of syphilis; one had involvement of the central nervous system, while the other had no gross evidence of the disease other than the nodules. The Wassermann test was positive in both. In one the lesion had been present about six months and in the other about three years before the date of biopsy. In size and location these nodules were identical with those from patients with rheumatoid arthritis.

All nodules were excised under local anesthesia, care being taken to inject the procaine hydrochloride around and not into the substance of the nodule. Half of each excised nodule was placed in fixing solution for the preparation of ordinary microscopic sections; the other half, freed from extraneous tissue, was used for supravitally stained preparations. Since the technic was identical with that previously described,[4] it will be sufficient to say that scrapings of the nodules were exposed to weak dilutions of neutral red and Janus green on

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Thornley and Dr. G. B. Rhodes, of the United States Marine Hospital 70, New York City.

specially prepared slides and were examined at 37 C. with the oil immersion lens.

RESULTS

All supravitally stained preparations showed small masses of tissue composed of interwoven cells and fibrils. Most of these masses were too dense for a suitable study, but lying among them were large numbers of individual cells which could be satisfactorily examined.

Rheumatic Nodules.—The results of a study of supravitally stained scrapings of rheumatic nodules, described in detail elsewhere,[4] need only be summarized here. With the exception of a few lymphocytes, plasma cells, monocytes and clasmatocytes, the cells encountered appeared to be of one type; they varied considerably, however, in size and shape. The cell membrane was indistinct in contrast to the sharp outline of the nuclear membrane. The cytoplasm and nucleus had an almost identical coarse ground glass appearance, against which one or two nucleoli stood out clearly. Mitochondria were not seen. A striking feature was the failure of the cells to take up neutral red, only a few cells showing from 2 to 4 small pale pink dots, which faded after about thirty minutes. A few small refractive bodies were commonly present. The predominating cell was roughly oval and measured from 20 to 30 microns long and from 15 to 20 microns wide; however, others twice as large as this type were not infrequent. Cells with 2 or 3 nuclei were occasionally seen, and one with 11. All gradations between these oval cells and long spindle-shaped elements were noted.

EXPLANATION OF FIGURE 1

A, low power view of a part of a rheumatoid arthritic nodule showing large areas of collagenous necrosis surrounded by highly vascular and cellular granulation tissue. Hematoxylin and eosin; × 27.

B, higher magnification of the portion of field marked in *A*, showing pronounced new vessel formation and cellular proliferation. Hematoxylin and eosin; × 75.

C, cross-section of a syphilitic nodule showing a central cavity, a thick layer of relatively acellular fibrous tissue and the surrounding areolar tissue. The necrotic material which originally filled the central cavity has been lost. Hematoxylin and eosin; × 5.

D, higher magnification of the area marked in *C*. The paucity of cells and vessels in the fibrous tissue comprising the nodule and the collections of cells in the loose perinodular tissue are illustrated. Hematoxylin and eosin; × 27.

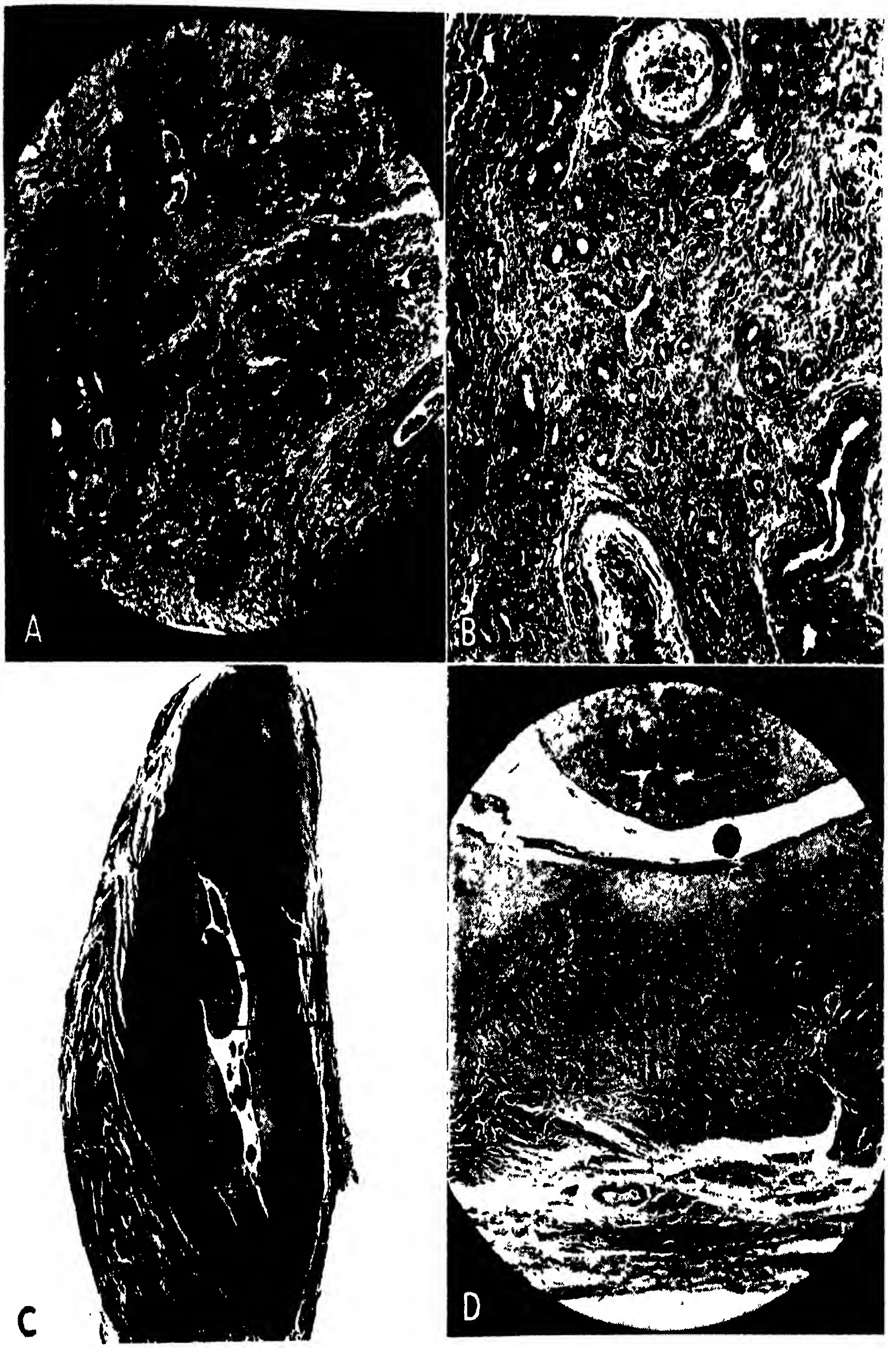

FIGURE 1

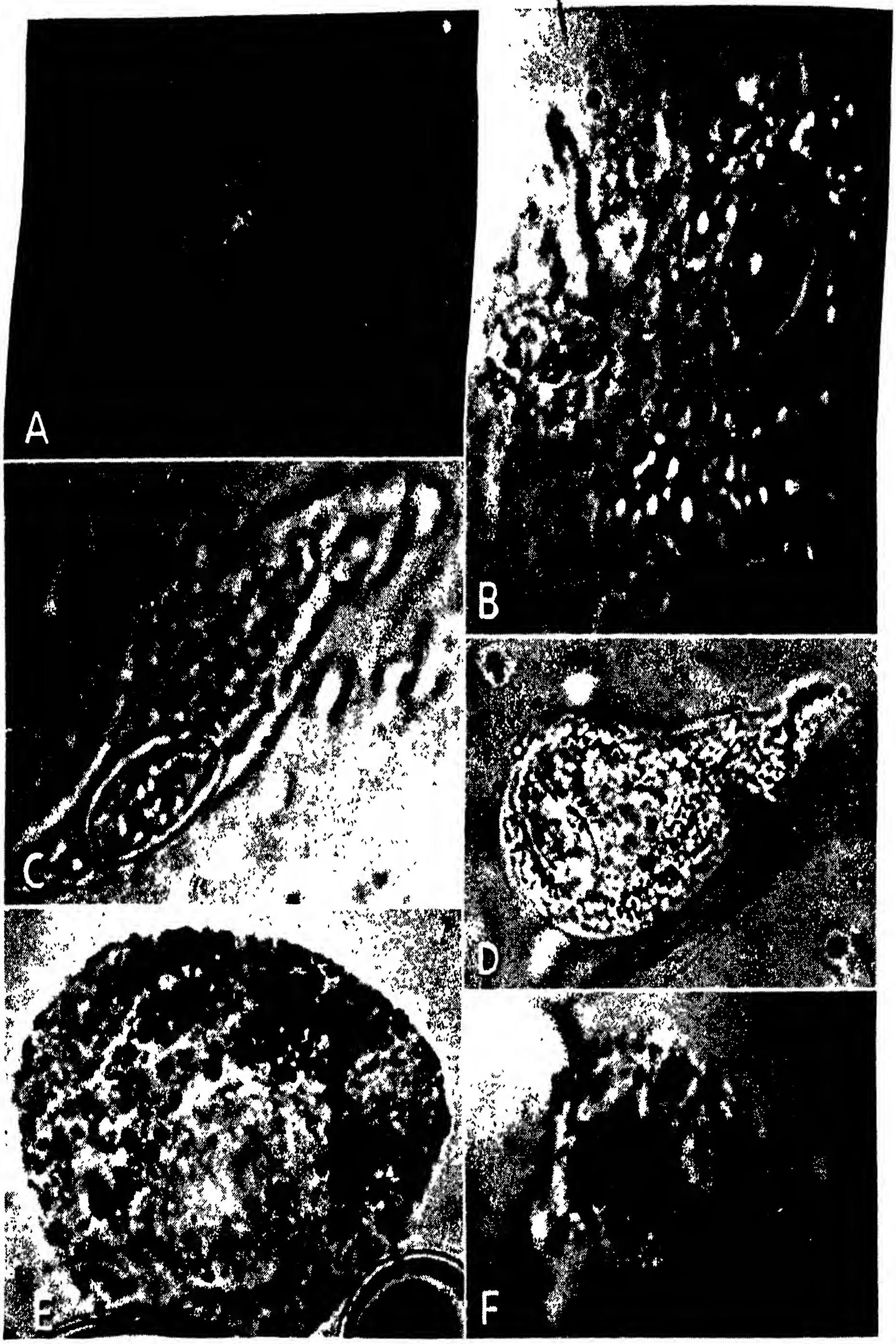

FIGURE 2

Cells having the general characteristics just described but no larger than intermediate lymphocytes were always present. These were thought to be primitive, undifferentiated connective tissue cells from which the others developed, or perhaps representative of an early stage of that differentiation. The cells of the predominant type were in all respects similar to those from a rheumatoid arthritic nodule, illustrated in figure 2*A*. Actual cells from rheumatic nodules are not shown here since drawings and photomicrographs of the various forms have already been recorded in paper I of this series.[4]

Rheumatoid Arthritic Nodules.—The gross appearance of the nodules from the eight patients with rheumatoid arthritis of this series was identical with that described by Dawson and Boots.[1f] In histologic sections the similarities between nodules of rheumatic fever and rheumatoid arthritis, which have been repeatedly described (Wick[1e] and others[2]) were corroborated. Characteristic photomicrographs are shown in figures 1*A* and *B*.

Examination of supravitally stained preparations of nodules from two patients with rheumatoid arthritis showed the cells to be both quantitatively and qualitatively identical with those of orthodox

EXPLANATION OF FIGURE 2

A, group of cells from a rheumatoid arthritic nodule, illustrating the sharp nuclear outline and nucleoli, the vague cytoplasmic detail and the failure to take up neutral red and Janus green. Nucleoli are beginning to stain from over-exposure to neutral red. A crenate erythrocyte is included for comparison of size. Supravitally stained; × 1,000.

B, cell from a rheumatoid arthritic nodule, containing large refractive bodies. Supravitally stained; × 1,700.

C, cell from a rheumatoid arthritic nodule, representing transition toward the long spindle-shaped cells. Supravitally stained; × 1,700.

D, multinucleated cell from a syphilitic nodule. The many small bodies which appear white in the photograph were uniformly orange-red in the original. Supravitally stained; × 1,700.

E, large cell from a syphilitic nodule. The entire cytoplasm is filled with dark and light bodies, which obscure the nucleus and which in the original were various shades of red and orange. Parts of two air bubbles are included in the illustration. Supravitally stained; × 1,700.

F, cell from a syphilitic nodule, presenting some features of both monocytes and clasmatocytes. The neutral red bodies are uniformly dark red and of fairly uniform size. No mitochondria were seen. Supravitally stained; × 1,700.

rheumatic nodules (fig. 2*A*). The cells of the remaining nodules were essentially the same, but minor variations were noted. In five instances, from 0.5 to 10 per cent of the cells contained large refractive bodies such as are shown in figure 2*B*, although usually smaller than those illustrated. These were never stained with neutral red but were glistening and tridimensional; possibly they represented a part of the process of degeneration. Another point of difference was the greater frequency of long spindle-shaped cells in the rheumatoid than in the rheumatic nodules; these were more than usually numerous in four of the nodules and in one comprised 30 per cent of the cells seen. Figure 2*C* illustrates an intermediate form of spindle-shaped cell, having the characteristics of the typical cells shown in figure 2*A* but definitely elongated and apparently developing in the direction of a fibrocyte. A third point to be noted was the presence of cells similar to the usual ones of rheumatic granulomas but containing small bodies which stained with neutral red. These were found in cells of only two nodules and as a rule were inconspicuous because of their minute size and pale color. They had the same appearance as the transiently staining bodies occasionally seen in cells of true rheumatic lesions. In one nodule 15 per cent of the cells seen contained from 2 to 20 of these minute neutral red bodies. In no instance were the latter larger than 0.5 micron in diameter, and when a number occurred in a given cell all were of the same size and color, which varied from pale pink to orange and usually faded to yellow or was lost entirely by the end of one or two hours. Cells containing these bodies were easily distinguished from monocytes and clasmatocytes, which were noted in small numbers in one nodule. Occasionally, lymphocytes and what were considered to be plasma cells were seen. In scrapings from five of the eight nodules, multinucleated cells in every way identical with those of the rheumatic nodules were identified. There appeared to be no correlation between the age of the nodules in this small series and the relative frequency with which spindle-shaped elements or cells containing refractive or neutral red bodies were found.

Syphilitic Nodules.—The syphilitic nodules were strikingly different from the rheumatic and rheumatoid lesions. They consisted of firm white capsules from 1 to 4 mm. thick surrounding yellowish necrotic material. In one case the mass excised was made up of five smaller

nodules closely bound together. Macroscopically these lesions appeared much less vascular than rheumatic and rheumatoid nodules. Microscopically they consisted of hyalinized connective tissue, which blended imperceptibly with the surrounding connective tissue fibers. At the center of each was a poorly defined cavity containing irregular hyalinized fragments possessing no definite structure. At the periphery of each nodule were collections of small round cells and larger cells containing brown pigment. The smaller blood vessels were moderately thickened but presented no characteristic features. The histologic structure distinguished these lesions from rheumatic and rheumatoid nodules, as has been pointed out by Crouzon and Bertrand[1e] and Dawson.[2f] Photomicrographs of one nodule are shown in figure 1*B* and *C*.

The findings in supravitally stained preparations also were strikingly different from those of rheumatic and rheumatoid nodules and were in general agreement with observations made on experimentally induced syphilitic lesions in rabbits. Morgan and Cunningham and their associates[7] studied supravitally stained scrapings of experimentally induced syphilitic lesions and found the predominant cells to be highly phagocytic large macrophages (clasmatocytes) containing many vacuoles of varying size and color. In similar studies Pearce and Rosahn,[8] on the other hand, found normal and stimulated monocytes in early lesions and clasmatocytes only later. In the two nodules which I examined, almost every cell took up neutral red in greater or less amount, and all cell types reported in experimental lesions were encountered. Normal, unstimulated monocytes were present in small numbers, but the predominant cells were of two types: (*a*) typical highly phagocytic clasmatocytes containing many vacuoles which varied in size and color (fig. 2*E*) and (*b*) cells containing smaller vacuoles of uniform size and color (fig. 2*F*). The color in the latter, although uniform within a given cell, varied from yellow to deep red in different cells, the predominant shade being a dark salmon. In some of these cells, as in the one illustrated, only a moderate number of

7. (*a*) Morgan, H. J.: Tr. A. Am. Physicians **45**:67, 1930. (*b*) Cunningham, R. S.; Morgan, H. J.; Tompkins, E. H., and Harris, S., Jr.: Am. J. Syph. **17**:515, 1933.

8. Pearce, L., and Rosahn, P. D.: Proc. Soc. Exper. Biol. & Med. **28**:654, 1931.

these neutral red bodies were seen, while in others they were so numerous that the nuclei were obscured; the cell membrane, in contrast, was always sharply outlined. One[1] cell with multiple nuclei was noted (fig. 2*D*). Whether these cells should be classified as clasmatocytes or as peculiar stimulated monocytes is uncertain, but they appear to be fairly characteristic of syphilitic lesions, as they occurred relatively frequently in the two human nodules described here, as well as in rabbit lesions placed at my disposal by Dr. Louise Pearce and Dr. P. D. Rosahn.

In the various preparations from the two human nodules a few small cells such as those found in rheumatic and rheumatoid granulomas were observed, but none of the characteristic large oval cells of the type predominating in the latter lesions, and no free spindle-shaped forms, although the latter were identified in the small tissue masses always present.

Controls.—Supravitally stained preparations of other subcutaneous lesions and tissues were examined as controls. These included a gouty tophus, a number of sebaceous cysts and normal human and rabbit subcutaneous tissue, fascia and tendons. In the study of the tophus and sebaceous cysts, care was taken to scrape only the fibrous capsules. The cells found in these scrapings were strikingly few compared with those from the granulomas. The preparations made from the tophus revealed merely a few spindle-shaped elements and small oval cells of the type noted in rheumatic and rheumatoid nodules and thought to be primitive, undifferentiated connective tissue cells. A very few of these small cells were found also in the scrapings of sebaceous cysts, but the chief cells present in the latter were clasmatocytes, basophils and lymphocytes. The normal tissues in supravitally stained preparations revealed merely a few spindle-shaped and small oval cells and large masses of apparently acellular fibrils. None of the characteristic large mononuclear or multinuclear cells of rheumatic and rheumatoid nodules was seen in any control tissue.

COMMENT

In this study, the predominant cells of syphilitic subcutaneous nodules differed strikingly from those of rheumatic and rheumatoid arthritic nodules in their reaction to supravital staining and proved

to be the distinctive stimulated monocytes and clasmatocytes previously shown[9] to be characteristic of syphilitic lesions. Thus, cytologic proof is added to the evidence provided by ordinary histologic study that the clinically similar subcutaneous nodules of rheumatoid arthritis and syphilis are pathologically dissimilar; the latter are shown to be not merely non-specific lesions but representative of syphilitic tissue reactions in general. This result gives indirect support to the belief that in rheumatic fever, too, the subcutaneous nodules are characteristic of granulomas elsewhere in the body and that conclusions drawn from a study of cells of the nodules are applicable also to those of the cardiac lesions.[4]

In contrast to the findings in the syphilitic lesions, the cells of nodules from patients with rheumatoid arthritis were in all essential features the some as those of orthodox rheumatic nodules. Obviously, this does not prove the identity of rheumatic fever and rheumatoid arthritis, but it does add one more bit of evidence to the clinical and histologic similarities suggesting a relationship between these two diseases, and, when taken into consideration with other histologic features, it indicates that at least in the proliferative phase of the tissue reaction they are similar.

Study of control material revealed that scrapings of normal connective tissues contained small numbers of the spindle-shaped cells and small oval cells noted in rheumatic granulomas. Probably the former are mature fibrocytes and the latter primitive, undifferentiated connective tissue cells, possessing, however, great capacity for differentiation in various functional directions when suitably stimulated. These small cells were more abundant in the zone of connective tissue reaction surrounding cysts and tophi, but although increased in numbers they failed to show differentiation into the large and sometimes multinucleated cells of rheumatic and rheumatoid granulomas. In a previous paper,[4] it was reported that scrapings of very early lesions of experimentally induced tuberculosis contain the same oval cells in abundance and, in addition, a moderate number of the larger cells noted in rheumatic granulomas; soon, however, these cells are converted into the monocytes and epithelioid cells characteristic of

9. Morgan.[7a] Cunningham and others.[7b] Pearce and Rosahn.[8]

tuberculosis. In rheumatic fever and rheumatoid arthritis, on the other hand, although the young connective tissue cells undergo marked increase in number and size and tend to develop into multinucleated forms, they show little or no differentiation so far as their capacity to react to neutral red and Janus green is concerned.

SUMMARY

A comparative study is reported of supravitally stained preparations of subcutaneous nodules from patients with rheumatic fever, rheumatoid arthritis and syphilis. The cells of rheumatoid arthritic nodules were found to have essentially the same characteristics as those of rheumatic fever. The cells of syphilitic nodules differed and proved to be those characteristic of syphilitic lesions in general. The bearing of these results on the nature of the characteristic cells of rheumatic granulomas in the heart and on the relationship between rheumatic fever and rheumatoid arthritis is discussed.

[Reprinted from COMPTES-RENDUS DES TRAVAUX DU LABORATOIRE CARLSBERG, 1938, Vol. 22, série chimique, pp. 480–486. JUBILEE VOLUME IN HONOR OF PROFESSOR S. P. L. SØRENSEN]

GASOMETRIC DETERMINATION OF CARBOXYL GROUPS IN AMINO ACIDS

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(Received for publication, October 16, 1937)

The first specific and general procedure for quantitative determination of the amino acids constitutes one of the debts owed by physiological chemistry to Sørensen, whose formol titration (4) is still one of the most useful and widely applied methods for studies involving the amino acids. Sørensen's titration was followed by other methods, such as the alcohol titration of Willstätter (11), and the nitrous acid method of Van Slyke (5). All of these methods, in the forms applied to biological material served as measures of the NH_2 or basic nitrogen groups of the amino acids (see discussion by Van Slyke and Kirk (7)).

For the other characteristic group of the amino acids, the carboxyl, there has been available only the method of Zirm and Benedict (12) which is a modification of the acetone titration of Linderstrøm-Lang (2). It yields excellent results with amino acid mixtures, but its application to biological material is limited by the fact that it includes more or less completely, according to their constants, all acid groups with dissociation constants in the range 10^{-2} to 10^{-5}, and consequently can not be applied to mixtures containing the common organic acids or phosphates (Van Slyke and Kirk (7)).

The present method is free from these limitations, and is suited to rapid and precise micro analyses. It utilizes the fact, noted by Ruhemann (3) and by Grassmann and von Arnim (1), that when amino acids react with ninhydrin evolution of CO_2 occurs. Under the conditions which we have utilized in the present determination, the CO_2 of the carboxyl groups of the alpha amino acids is quantitatively liberated in 3 minutes. The Van Slyke-Neill manometric apparatus (8), equipped as by Van Slyke, Page, and Kirk (9) for wet carbon combustions, is used for evolving and measuring the CO_2. A complete determination requires about 15 minutes.

Of the amino acids yielded by protein hydrolysis, each evolves exactly or approximately one mole of CO_2, except aspartic acid, which yields nearly two.

The reaction is sharply quantitative with all types of amino acids yielded by protein hydrolysis except aspartic, glutamic, and lysine. Aspartic acid evolves approximately 1.9 moles of CO_2 and glutamic acid 1.03. With lysine, quantitative evolution of one mole of CO_2 is obtained, but is followed by a slow evolution of more CO_2, so that it is necessary to limit the boiling with ninhydrin exactly to the specified time in order to obtain accurately one mole of CO_2. With amino acids other than these three, one mole of CO_2 is evolved quickly and completely, and further boiling does not change the results. Proline and hydroxyproline react in the same manner as the amino acids with primary NH_2 groups. The results are sufficiently exact to be used as criteria of analytical purity of the individual amino acids.

Ordinary organic acids, such as acetic, lactic, and citric, evolve no CO_2; urea also evolves none.

Peptides evolve no CO_2, even from their free carboxyl groups. This reaction with ninhydrin therefore provides a quantitative measurement of free amino acids in the presence of peptides. Such differentiation is not provided by the other methods for NH_2 and COOH estimation previously mentioned, since they measure the free NH_2 or COOH groups in peptides as well as in free amino acids.

Molecular structures which provide carboxyl groups yielding CO_2 quantitatively under the conditions of the analysis are

$$\overset{\text{I}}{\text{R—}\underset{\displaystyle\text{NH}_2}{\underset{|}{\text{CH}}}\text{—COOH}} \quad \text{and} \quad \overset{\text{II}}{\text{R—}\underset{\displaystyle\text{HNCH}_2\text{R}}{\underset{|}{\text{CH}}}\text{—COOH}}$$

Of structure I are the amino acids with primary alpha NH_2 groups. Of structure II are proline, hydroxyproline, and monomethyl glycine (sarcosine).

If the NH_2 is moved away from the COOH, from the alpha position to the beta or gamma position, the reactivity of the carboxyl CO_2 diminishes, but may not entirely disappear. Thus in aspartic acid, evolving 1.9 moles of CO_2 from the two carboxyl groups, the COOH to which the NH_2 is beta presumably yields 0.9 mole. In beta alanine the carboxyl is less reactive, yielding about 0.1 mole of CO_2. In

glutamic acid, of the 1.03 moles of CO_2 evolved, presumably only 0.03 are from the carboxyl gamma to the NH_2.

The following types of amino acid derivatives yield no CO_2 under the conditions of the analysis:

1. Derivatives in which a H of the NH_2 group is replaced by COR. Such are peptides and acetylated or benzoylated amino acids.

2. Derivatives which have no H atom on the amino nitrogen. Thus, while monomethyl glycine (sarcosine) evolves a mole of CO_2, dimethyl glycine (with both methyl groups on the N) evolves none.

3. Derivatives in which the free COOH group is substituted by an ester or amide group.

The reaction of ninhydrin with the amino acids occurs with the formation of blue colored products, and the color formation has been used for detection and semi-quantitative estimation. Ruhemann (3) the discoverer of the color reaction, and Grassmann and von Arnim (1), have discussed the probable mechanism of the reactions involved in the formation of both color and CO_2. That the CO_2 evolution does not necessarily accompany or follow the color formation, however, is shown by our results with peptides. They yield the color, but not the CO_2.

Apparatus

The apparatus consists of the Van Slyke-Neill manometric apparatus (8) and the accessory parts used for the determinations of carbon in organic compounds as described by Van Slyke, Page, and Kirk (9). The arrangement is indicated in Figure 1. In addition, there is needed a 1.5 cc. stopcock pipette, and appropriate storage vessels for 5 N sodium hydroxide and for CO_2-free 0.5 N sodium hydroxide. The alkali storage tube *N*, with soda-lime protection shown in the upper part of Figure 1 is convenient.

Reagents

Ninhydrin (Triketohydrindene Hydrate)[1]. We have used the preparation made by Eastman Kodak Co. It is used in the solid form.

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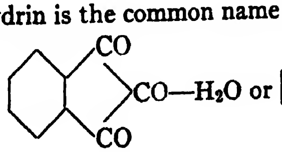
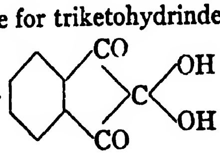
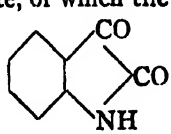
[1]) Ninhydrin is the common name for triketohydrindene hydrate, of which the structure is  or . Isatin,  also reacts with the amino acids with evolution of CO_2. and can be used for

Citric Acid Buffers. For the ninhydrin reaction in aqueous medium, two citric acid buffers are used. One, for pH 4.7, consists of a mixture of 17.65 gms.

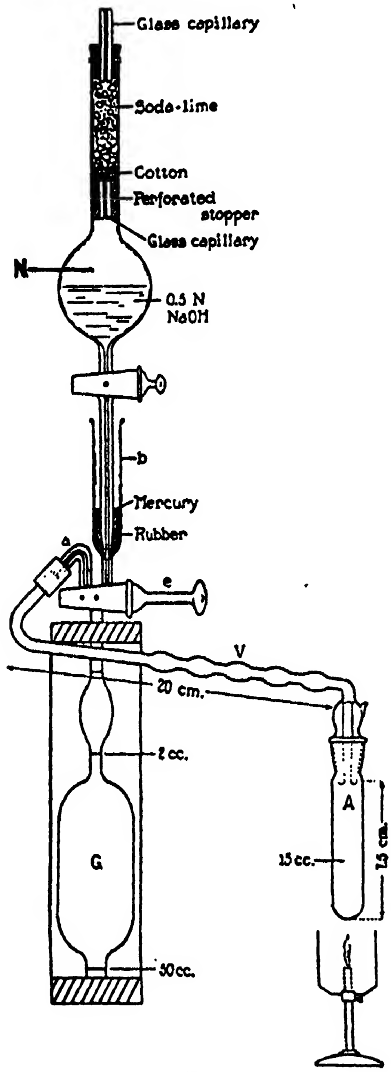


FIG. 1

of trisodium citrate ($Na_3C_6H_5O_7 \cdot 2\ H_2O$) and 8.40 gms. of citric acid ($C_6H_8O_7 \cdot 1\ H_2O$). The other, for pH 2.5, consists of 2.06 gms. of trisodium citrate and 19.5 gms. of citric acid. In preparing the buffers the trisodium citrate and citric

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carboxyl determination with the same technique as ninhydrin. The reaction with isatin, however, is slower than that with ninhydrin, which is the preferable reagent, at least for use in aqueous solutions.

acid are both finely ground separately in an agate mortar and are then mixed in the correct proportions. After mixing they cake appreciably, but the cake is easily broken up by two subsequent grindings of the mixtures in the mortar. Thereafter, they remain powderlike. Both buffers are used in the solid form.

Approximately 0.5 N NaOH of minimal CO_2 content. To 100 cc. of freshly boiled water containing a drop or two of 0.1 N HCl are added 3.2 cc. of clear, concentrated NaOH (1 gram NaOH to 1 cc. of water), prepared approximately carbonate-free by letting any carbonate settle. The 0.5 N solution is immediately transferred to a storage vessel which provides protection from atmospheric CO_2 (vessel *N*, Figure 1).

Approximately 5 N NaOH. This is prepared from the concentrated 1:1 NaOH solution by diluting 1 volume to 3 volumes with water.

Approximately 1 N Lactic Acid. Concentrated lactic acid (sp. gr. 1.20) is diluted, 1 volume to 10 volumes, with water.

Procedure

Preparation of the Samples for Analysis. Samples should preferably contain 0.3 to 0.6 mg. of carboxyl carbon, when the CO_2 pressure is to be measured with the gas at 2 cc. volume. The reaction is carried out in a liquid volume of approximately 1.0 cc., so if convenient, the sample is dissolved (tube *A*) in this volume of water. Three alundum beads[1]) to prevent bumping and 50 mg. of citrate buffer are then added and the solution is boiled for a moment to remove any CO_2 that may be present. For lysine, buffer of pH 2.5 must be used. For the mono amino acids pH 4.7 is employed. For the arginine and histidine either 2.5 or 4.7 may be used. Lysine gives results at pH 4.7 which are 10–15 per cent. too high.

Reaction with Ninhydrin. Exactly 2.00 cc. of 0.5 *N* CO_2-free NaOH are admitted to the mercury-filled extraction chamber *G* from the alkali tube, *N*, which is shown in Figure 1. The precautions described by Van Slyke, Page, and Kirk to avoid contamination with CO_2 from the atmospheric air, are followed. The alkali tube is removed, and the clean, dry connecting tube *V* is attached to *G*. To the amino acid sample in the combustion tube *A* are added 50 mg. of ninhydrin. A thin layer of glycerine is applied to the glass joint of the combustion tube *A* by means of a glass rod dipped into a bottle of syrupy glycerine. This serves as a seal when the combustion tube is attached. The connection of *A* with *V* is made immediately after the application of the glycerine. Stopcock *e* is turned to connect *A* to *G*, the Hg surface in the reservoir being slightly below the level of chamber *G*, so that the system is under slightly reduced pressure.

A micro burner is placed under *A* and the solution is brought quickly to a boil.

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[1]) "Alundum grains, black-grain size 14", manufactured by the Norton Co., Worcester, Massachusetts, have been used.

It is then gently boiled for exactly three minutes. During this interval the water vapor condenses in the connecting tube V and runs back into A. The heating is carefully regulated so that water vapor does not pass beyond the upper end of V. Failure to observe this precaution would bring about a loss of too much liquid from the combustion tube, and consequent danger of charring; it would also increase the amount of solution in G, so that its exact volume would not be that assumed in the calculations to be made later.

Transfer of CO_2 to alkali solution in G. After the initial 3-minute heating the micro burner is lowered several centimeters below the bottom of A and the gas is passed back and forth six times between A to G by raising and lowering the mercury reservoir. Each time the mercury bulb is lowered the liquid in the combustion tube boils vigorously, particularly the first two or three times. However, if the micro-burner is lowered sufficiently and the mercury bulb is not kept at the low level too long for the first passage, no liquid boils over into G. After six passages stopcock e is closed while the mercury bulb is at a level such that approximately 20 cc. of gas space is in G. The gas thus segregated in G is freed from the last traces of CO_2 by raising and lowering the mercury six times. The unabsorbed gas is ejected through b and stopcock e is sealed with mercury.

To transfer the last portions of CO_2 to G from the gas and liquid in A, the mercury in G is lowered about half way to the 50 cc. mark[1]), and A and G are connected by turning cock e. During gentle heating of the combustion tube the remaining CO_2 is absorbed in the alkali of the chamber by six more passages of the remaining gas from A to G and back. The gas phase in G is finally reduced to about 0.5 cc. by raising the leveling bulb, and stopcock e is closed. The connecting tube V is removed, and the capillary a is filled with Hg from the outside. The gas remaining in G is ejected through b. About 1 cc. of Hg is then poured into the cup and 1.5 cc. of 1 N lactic acid are admitted into the chamber by means of a stopcock pipette, which is introduced into cup b in the same manner shown for alkali tube N in Figure 1. The CO_2 liberated by the acid is extracted from the solution by shaking at reduced pressure as in determination of CO_2 in blood (6). The gas pressure p_1 is measured with the gas at 2 cc. volume. The CO_2 is then absorbed with 0.5 cc. of 5 N NaOH and the p_0 reading is made with the unabsorbed gas at 2 cc. volume.

Blank Analyses—"c" correction. The "c" correction is the CO_2 formed in a blank analysis, with the reagents present, but no amino acid. The ninhydrin may also be omitted from the procedure, since in no case has ninhydrin been found to contribute to the c correction. Omission of the ninhydrin from the blanks results in an appreciable saving of this expensive reagent. However, before relying on blanks in which the ninhydrin is omitted, its lack of effect should be checked with the particular reagents in use.

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[1]) If the pressure in G is not reduced, mercury will rush from G into V when cock a is opened.

Calculations

The pressure P_{CO_2}, of CO_2 from amino acid carboxyl groups, is calculated as:

$$P_{CO_2} = p_1 - p_0 - c$$

where c is the pressure difference $p_1 - p_0$, observed in a blank determination. The carboxyl carbon is calculated as $P_{CO_2} \times$ *factor* where the factor is from Table I. Table I is computed from the tables of Van Slyke and Sendroy (10) for the "*a*" volume of 2 cc., S volume of 3.5 cc., and temperature indicated.

TABLE I

Factors by Which P_{CO_2}, Measured with CO_2 Gas at 2 CC. Volume, Is Multiplied to Obtain Milligrams of Carboxyl Carbon

Temperature °C	Factor	Temperature °C	Factor
10	0.001519	25	0.001398
11	09	26	91
12	00	27	85
13	0.001491	28	78
14	82	29	72
15	74	30	65
16	66	31	59
17	58	32	53
18	50	33	48
19	42	34	42
20	35	..	..
21	27	..	..
22	20	..	..
23	12	..	..
24	05	..	..

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CHANGES WITH AGE IN THE BASAL METABOLIC RATE IN ADULT MEN[1]

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That alterations in physiological behavior are associated with growth of the body is an obvious biological event. The measurement of such alterations at different periods of life, their increase or decrease, and the rate of these changes—whether rapid or slow—has not been frequently undertaken especially in late adult life.

To add to knowledge of the behavior of the body after age 40 is becoming increasingly important, because of the increase of the population in the older decades and, therefore, the greater number of affections in the aged. For this reason to possess analyses of physiological changes between the 7th and the 9th decades has become desirable, for changes barely detectable in the 5th and 6th have then advanced so as to be clear and measurable. The meaning of the changes, noticed earlier, for the subsequent development of chronic degenerative diseases, can then be appreciated. To be able to make correct descriptions, measurements illustrative of these changes should be collected in an orderly and systematic fashion. In the light of the results, correlations indicative of the interdependences of various organs, as for example of kidneys, heart and lungs, can then be undertaken.

The study now reported has proceeded from this point of view. It is concerned with the period of human life after the fortieth year. The subjects were men, apparently healthy. The functions observed have been 1, the metabolism of the body in terms of the consumption of oxygen; 2, the volume of blood put out by the heart per minute, in association with certain related functions; 3, the blood pressures; 4, the ability of the kidneys to remove the products of nitrogenous metabolism from the blood, and to concentrate the urine; 5, the amount of certain substances in the blood related to the activity of the kidneys and to the state of the arteries; 6, the vital capacity of the lungs; 7, the electrocardiograms; and 8, the state of the heart, great, and long arteries as shown in roentgenograms.

[1] An abstract of these studies was read before the American Society for Clinical Investigation, April 30, 1934.

TABLE 1

Number of measurements of basal metabolism in normal subjects over 40 years of age previously reported

REPORT NO.	DATE	AUTHOR	SEX	NUMBER OF SUBJECTS BY AGE GROUP: 40 to 44	45 to 49	50 to 54	55 to 59	60 to 64	65 to 69	70 to 74	75 to 79	80 to 84	85 to 89	90 to 94	95 to 99	106	Sum	Compare with Report No. 3	Compare with Report No. 4	NOTES
1	1899	Magnus-Levy and Falk (23)	♂	1			1	1		2	2						7			
2	1916*	Du Bois (8)	♂	4			1										5			
3	1917*	Aub and Du Bois (5)	♂								3	3					6	+7%		Cf. with Report No. 2
4	1919	Harris and Benedict (7)	♂	5	1		2	3									11	−3 to −4%		
			♀	4	3	6	4	3	1	2							23			
5	1920*	Dreyer (24)																+1.1%		
6	1921*	Blunt and Dye (25)	♀	3														−2.1%	−0.8%	
7	1922	Boothby and Sandiford (26)	♂	3	2	2	4	1									12	+1.0%	+5.7%	
			♀	4	3	7	2	1	1								18	+0.3%	+4.2%	
8	1923*	Krogh (10)																−4 to −6%		
9	1925*	MacLeod and Rose (27)	♀	13														−12.9%	−6.8%	
10	1928*	Benedict (28)	♂	4		1		2					1				8	−4.4%	−1.8%	
			♀	3	2	4	1										10	−7.3%	−4.2%	
11	1929*	Boothby and Sandiford (29)	♂	1822	See note													−1 to −4%		
			♀	5066																
12	1929*	Wolf (30)	♂							2			1				3	−6.8%	+8.3%	
			♀								1						1	+3.2%	+9.0%	
13	1932*	Benedict and Meyer (31)	♀						2	9	4	7	1				23			
14	1932*	Albergo (32)	♂	1				2	8	4	3	4	1				23			
15	1932*	Lucchi (33)	♂			3	1	6	8	9	2	2					31			
			♀					2	2	2	1						7			
16	1934*	Kise and Ochi (34)	♂			5	8	7	8	4	6	4	1	1			44	Slightly lower	Slightly higher	
			♀			2	6	4	12	4	14	7	1				50			

17	1934*	Benedict and Root (35)	♂											1			1	−24.0%	−4.5%	
18	1934*	Matson and Hitchcock (36)	♂							1	5	3	4	1			14	+6.9%	−13.6%	
			♀								1	4	1	1		1	8	−16.4%	−11.6%	
19	1935*	Benedict (21)	♂							2		1	2				5	−10.7%	+3.1%	
			♀						1	3	3	1	2				10	−8.2%	+0.2%	
20	1936*	Boothby, Berkson, and Dunn (20)	♂	61	42	41	26	13									183	?1.3% lower		Cf. with Report No. 11
			♀	64	49	64	41	18	15								251			

* Explanations. (The numbers correspond to the reports.)

2. Curve of metabolism from birth to 80 years, using data on subjects over 40 years of age, based on data in nos. 1 and 2.
3. Subjects were inmates of institutions—not active, and showed stigmata of senility. Curve given from birth to 80 years, using data on subjects over 40 years of age based on data in nos. 1, 2 and 3.
5. Equations for men and women, using data on subjects over 40 years of age, based on data in nos. 1, 2, 3, 4.
6. Included in report on 17 normal women, aged 21 to 44 years.
8. Curves from 15 to 70 years, based on data in nos. 1, 2, 3, 4.
9. Included in report on 92 normal women, aged 20 to 49 years.
10. Records of second normal series from his laboratory; 27 men, aged 21 to 89 years, average 34 years; 33 women, aged 18 to 58 years, average 32 years. Benedict calls attention to distinction between "hospital normal" and physiological standards, the use of the former being justified for clinical purposes only.
11. Curves for men and women from 5 to 70 years, extrapolated to 70 to 80 years. No age distribution reported.
12. Normal subjects.
13. Individual records and curves given in Benedict (1935).
14. Italian subjects. Results given in calories per square meter of body surface per hour with record of body size. Percentage deviations given from Aub-Du Bois table of 40 calories per hour per square meter in young adult men.
15. Italian subjects. Results given in calories per square meter per hour. No records of body size. No significant deviations from prediction values of Harris and Benedict tables.
16. Japanese subjects. Curves for men and women from 50 to 90 years.
17. One normal subject. Includes records of 2 normal men previously reported, one by Benedict, 1928, one by Wolf, 1929.
18. Inmates of institution—not active. Subject of 106 years (birth record not obtained)bed-ridden for 3 years.
19. Subjects observed under home surroundings. In good health and active.
20. Subjects and patients of the Mayo Clinic, selected clinically as being free from metabolic disturbance. Curve and nomogram for men from 6 to 64 years; for women from 6 to 69 years. Boothby, Berkson and Dunn, (1936) include data on 13 men, 60 to 64; and 33 women, 60 to 68 years (their oldest groups).

There were one hundred men, between ages 40 and 90 years, twenty in each decade, and besides, two men, aged 91 years and one, 101 years. All were physically active; none was an inmate of an institution.

Plan of observations. Each subject was admitted for observation for two days. During the first, the history was taken, the physical examination was made, and the clearance of urea by the kidneys was measured. A rehearsal in the procedures employed in estimating the basal metabolism and the cardiac output was undertaken. On the morning of the second day, these functions and the blood pressures were measured under basal conditions—the subject at rest, after the night's sleep and fourteen hours after the last meal. Blood for analysis of lipid and calcium content was obtained during the fasting state. Arterial blood for estimation of oxygen saturation was collected under oil from the radial artery. The electrocardiograms and the x-ray photographs of the heart, lungs and extremities were taken. The conditions surrounding the men studied in these reports were unusual. They were in hospital and had been there over-night. If the levels of measurement obtained were lower than is usual, it must be due to a state of relaxation of these men. Relaxation is not necessarily the ideal state. It marks a low level of activity, not necessarily the lowest, between which and various degrees of exertion, the consequences of exertion can be observed. To be observed at the low level of relaxation has the value of permitting estimates to be made of effects entailed by work.

The basal metabolic rate. The basal metabolic rate of uniform groups representative as were these of the five decades beginning with the 5th, has not before been studied. After age 70, the number of measurements so far reported is limited (table 1). Even so, as will appear, although the number has been sufficient to learn that the general course of the curve during these decades is downward, it has been insufficient to describe its form in detail decade by decade. The Benedict-Roth universal apparatus was used in most of the cases; the Sanborn apparatus in the rest. The average of two tests was regarded a correct measurement if the two agreed within 6 per cent (1). If the respiratory quotient is regarded as being 0.82, as is permissible, the error of the measurement does not exceed 1 to 2 per cent (2, 3, 4). Throughout adult life, the quotient undergoes no significant change (5, 6).

The calculations of the basal metabolic rate of normal individuals may be presented in two ways. Age, sex, height, enter, of course, as factors into the calculation. The difference in the curves between Harris and Benedict and Du Bois lies in an advantage which Benedict gave to weight—weight having a value, so far as the basal metabolic rate is concerned, greater than the other components in the calculation. This procedure has been usual. Both Harris and Benedict (7) and Du Bois (4, 8) have

taken these factors into account. There is a difference between them however in that Du Bois uses the area of the body surface as a proportional, but not causal, measurement of the metabolism, and that Benedict predicts the metabolism from body mass, calculated from an equation based on direct physical measurements and considered to be not only a proportional but also a causal measurement of the metabolism.

Inasmuch as the former uses height and weight in the calculation of surface area and the latter in the calculation of body mass, the practical differences due to the slightly different height-weight factors are small (9, 10). More marked discrepancies in their curves are due to differences in values attributed to age and sex as factors (9).

THE RESULTS. Each of several aspects[2] of the results deserves attention.

a. It is, of course, well known that in a given class of individuals the results of measuring the metabolic rate are scattered over a range sometimes of considerable extent. Of a group selected according to specified criteria, a single figure may not, for this reason, be regarded as descriptive of each member. Actually, no such figure exists. An average may be ascertained and the deviations from that average of all the individuals. The deviations are to be regarded as being equally important as is the average. To discover a scattering, a range of measurements, is in any case the usual experience. That this is a fact deserves and indeed requires recognition. The measurements which have been obtained illustrate this common experience (fig. 1, table 2). In the age group 45 to 49 years, the several deviations from the mean ranged from −15.1 per cent (30.2 calories per square meter per hour in a Y.M.C.A. secretary) to +10.8 per cent (39.2 calories in a Captain in the New York Fire Department). At other age levels similar scattering occurred. In the low range in a younger group, cases are therefore found at the same level as in the high range of an older one. At age 82 an active retired Colonel, U. S. Army, (number LXXXVII) for example, exhibited a rate of 38.2 calories while at age 72, a president of a large New York department store (number LXX), one of 28.1 calories. And yet the rate of the oldest man studied (101 years) was only slightly less than this, though his vigor was on a distinctly lower plane.

b. In a large population exhibiting a wide age span, the metabolic rate varies conceivably with age. There are in fact data, already published, which indicate that this is a fact. A single average figure is, therefore, undoubtedly not descriptive of the metabolic level of the entire population.

It is apparent that the level of the basal rate undergoes a progressive decline (fig. 1) with advancing age and that the decline is described by a

[2] These aspects (a b c d) of this study are still under investigation.

TABLE 2

Measurements of basal metabolism taken of the men examined

GROUP	SUBJECT NO.	AGE†	HEIGHT	BODY WEIGHT (WITHOUT CLOTHING)	BODY SURFACE AREA (DU BOIS WEIGHT-HEIGHT CHART)	OXYGEN CONSUMPTION, 0°C., 760 MM. HG.	METABOLISM: Total (Calories per 24 hours)	METABOLISM: Per kgm. (Calories per 24 hours)	METABOLISM: Total (Calories per hour)	METABOLISM: Per sq. meter body surface (Calories per hour)	DIFFERENCE FROM CURVES OF: Harris Benedict (1919)	DIFFERENCE FROM CURVES OF: Du Bois Boothby Sandiford (1929)
		yrs.	*cm.*	*kgm.*	*sq. meter*	*cc.*					*per cent*	*per cent*
40–44	I	40	174.0	83.6	1.98	257	1788	21.4	74.5	37.6	−1.5	−1.8
	II	40	164.0	64.2	1.68	228	1579	24.6	65.8	39.2	+5.3	+2.3
	III	41	174.0	66.4	1.79	263	1831	27.6	76.3	42.6	+16.3	+10.1
	IV	41	175.0	71.7	1.85	253	1757	24.5	73.2	39.6	+6.4	+3.3
	V	41	175.5	70.8	1.85	238	1654	23.4	68.9	37.2	+0.8	−3.0
	VI	43	178.0	70.1	1.85	233	1622	23.1	67.6	36.5	−0.5	−4.9
	VII	44	165.5	56.5	1.60	204	1421	25.2	59.2	37.0	+3.6	−3.5
	VIII	44	163.0	54.2	1.57	191	1325	24.4	55.2	35.2	−0.4	−8.8
	IX	44	175.0	98.7	2.14	251	1747	17.7	72.8	34.2	−12.7	−12.0
	X	44	179.5	70.8	1.88	219	1522	21.5	63.4	33.7	−7.2	−13.6
			Average		1.819				Average		+1.01	−3.2
45–49	XI	45	175.5	63.7	1.77	217	1508	23.7	62.7	35.4	−0.5	−6.8
	XII	45	180.0	70.6	1.88	243	1688	23.9	70.3	37.4	+3.3	−1.1
	XIII	45	172.0	80.1	1.93	201	1397	17.4	58.2	30.2	−19.0	−25.0
	XIV	46	171.0	60.7	1.69	217	1508	24.8	62.8	37.2	+4.3	−1.6
	XV	46	173.5	75.0	1.88	234	1626	21.7	67.8	36.1	−1.8	−4.7
	XVI	47	180.0	70.2	1.88	219	1522	21.7	63.4	33.7	−5.8	−12.2
	XVII	47	181.0	74.6	1.94	263	1827	24.5	76.1	39.2	+8.8	+3.6
	XVIII	48	165.0	55.4	1.59	214	1487	26.8	62.0	36.0	+11.9	−5.0
	XIX	48	177.5	76.2	1.93	231	1605	21.1	66.9	33.6	−4.3	−12.5
	XX	49	169.0	57.3	1.65	207	1440	25.1	60.0	36.9	+5.2	−2.4
			Average		1.816				Average		+0 21	−6.8
50–54	XXI	50	179.0	78.5	1.96	205	1424	18.1	59.3	30.3	−16.4	−18.5
	XXII	51	177.5	70.7	1.87	246	1709	24.2	71.3	38.1	+8.0	+2.4
	XXIII	51	173.0	56.5	1.65	212	1473	26.1	61.3	37.2	+8.0	0
	XXIV	51	161.0	69.0	1.72	198	1376	19.9	57.4	33.4	−6.8	−10.2
	XXV	51	165.0	65.0	1.71	195	1358	20.9	56.6	33.2	−5.8	−10.8
	XXVI	53	169.5	70.4	1.80	221	1536	21.8	64.1	35.6	+0.7	−4.3
	XXVII	53	175.0	75.8	1.90	226	1570	20.7	65.4	34.4	−3.5	−7.5
	XXVIII	53	169.0	47.5	1.52	192	1334	28.1	55.5	36.5	+10.4	−1.9
	XXIX	53	172.0	81.6	1.94	288	2001	24.5	83.3	42.9	+18.3	+15.3
	XXX	54	171.0	59.0	1.68	226	1570	26.6	65.3	38.9	+14.7	+4.6
			Average		1.775				Average		+2.8	−3.1
55–59	XXXI	55	179.0	77.0	1.94	209	1452	18.9	60.5	31.2	−11.9	−14.8
	XXXII	55	175.5	61.0	1.74	203	1410	23.1	58.8	33.8	−0.1	−7.7
	XXXIII	55	176.5	80.0	1.96	222	1542	19.3	64.3	32.8	−8.1	−10.4
	XXXIV	56	178.0	58.0	1.72	177	1230	21.2	51.3	29.8	−10.6	−18.6
	XXXV	57	163.5	76.5	1.81	222	1542	20.2	64.3	35.5	−0.6	−3.0
	XXXVI	58	177.5	61.8	1.77	201	1397	22.6	58.3	32.9	−1.1	−10.0
	XXXVII	58	172.0	71.6	1.83	194	1348	18.8	56.2	30.2	−11.3	−17.5

TABLE 2—*Continued*

GROUP	SUBJECT NO.	AGE†	HEIGHT	BODY WEIGHT (WITHOUT CLOTHING)	BODY SURFACE AREA (DU BOIS WEIGHT-HEIGHT CHART)	OXYGEN CONSUMPTION, 0°C., 760 MM. HG.	METABOLISM: Total (Calories per 24 hours)	METABOLISM: Per kgm. (Calories per 24 hours)	METABOLISM: Total (Calories per hour)	METABOLISM: Per sq. meter body surface (Calories per hour)	DIFFERENCE FROM CURVES OF: Harris Benedict (1919)	DIFFERENCE FROM CURVES OF: Du Bois Boothby Sandiford (1929)
		yrs.	*cm.*	*kgm.*	*sq. meter*	*cc.*					*per cent*	*per cent*
55–59 *Cont'd*	XXXVIII	59	178.0	76.6	1.90	258	1793	23.4	74.8	39.4	+11.2	+7.7
	XXXIX	59	171.5	72.8	1.84	184	1278	17.6	53.3	29.0	−16.3	−20.8
	XL	59	178.5	87.6	2.06	245	1702	19.4	70.8	34.4	−3.6	−6.0
			Average		1.857				Average		−5.2	−10.1
60–64	XLI	60	169.0	65.7	1.74	220	1529	23.3	63.8	36.7	+8.4	+1.9
	XLII	60	172.5	61.8	1.72	202	1403	22.7	58.6	34.7	+2.2	−3.7
	XLIII	61	166.5	67.4	1.74	211	1466	21.8	61.2	35.7	+3.8	−0.8
	XLIV	61	171.5	71.8	1.83	230	1598	22.3	66.7	36.4	+6.5	+1.1
	XLV	61	173.0	67.0	1.78	215	1494	22.4	62.2	34.9	+3.6	−3.2
	XLVI	61	179.0	91.0	2.09	237	1647	18.1	68.7	32.9	−8.6	−9.4
	XLVII	63	173.0	87.2	2.00	257	1786	20.5	74.4	37.2	+4.7	+3.2
	XLVIII	63	172.0	71.0	1.82	208	1445	20.4	60.2	33.1	−2.2	−8.7
	XLIX	64	172.5	74.8	1.86	226	1570	21.0	65.4	35.2	+3.0	−2.3
	L	64	174.0	54.4	1.65	188	1306	24.0	54.4	32.1	+4.2	−12.1
	LI	64	167.5	51.7	1.57	187	1299	25.1	54.1	34.5	+9.9	−4.3
	LII	64	164.0	76.6	1.82	205	1424	18.6	59.2	32.5	−5.6	−10.8
	LIII	64	183.0	58.2	1.75	193	1341	23.0	55.9	31.9	−0.7	−12.8
			Average		1.798				Average		+2.2	−4.8
65–69	LIV	65	174.0	80.0	1.94	239	1661	20.8	69.2	35.7	+3.9	+1.0
	LV	65	165.0	63.2	1.68	195	1355	21.4	56.4	33.4	+2.5	−5.7
	LVI	66	170.5	67.6	1.77	233	1619	23.9	67.4	38.1	+15.4	+7.4
	LVII	66	168.5	61.6	1.69	218	1515	24.6	63.2	37.4	+15.6	+5.6
	LVIII	67	182.0	87.8	2.09	249	1730	19.7	72.1	34.5	−0.1	−2.3
	LIX	68	170.0	51.0	1.58	214	1487	29.2	61.9	39.1	+28.3	+9.7
	LX	69	167.5	57.3	1.64	205	1424	24.9	59.2	36.1	+16.2	+2.2
			Average		1.770				Average		+11.7	+2.6
70–74	LXI	70	169.5	66.2	1.75	212	1473	22.3	61.3	35.0		(+0.6*
	LXII	70	175.5	80.5	1.96	239	1661	20.6	69.2	35.3		+1.4
	LXIII	70	153.0	59.0	1.55	169	1174	19.9	49.0	31.6		−11.3
	LXIV	71	168.0	84.0	1.93	245	1702	20.3	71.0	36.8		+5.4
	LXV	71	160.0	61.0	1.63	193	1341	22.0	56.0	34.4		−1.1
	LXVI	71	161.0	57.5	1.59	192	1334	23.2	55.5	34.9		+0.3
	LXVII	71	166.0	56.8	1.62	196	1362	24.0	56.8	35.1		+0.9
	LXVIII	72	172.0	81.2	1.94	239	1661	20.4	69.3	35.7		+2.5
	LXIX	72	165.0	48.6	1.52	191	1327	27.3	55.2	36.3		+4.1
	LXX	72	176.0	69.5	1.84	179	1244	17.9	51.7	28.1		−23.8
	LXXI	72	164.5	57.2	1.62	200	1390	24.3	57.9	35.7		+2.5
	LXXII	73	171.0	80.8	1.92	248	1723	21.3	71.7	37.3		+6.7
	LXXIII	74	172.5	68.6	1.804	193	1341	19.5	56.0	31.1		−11.9
			Average		1.744				Average			−1.8

TABLE 2—*Concluded*

GROUP	SUBJECT NO.	AGE†	HEIGHT	BODY WEIGHT (WITHOUT CLOTHING)	BODY SURFACE AREA (DU BOIS WEIGHT-HEIGHT CHART)	OXYGEN CONSUMPTION, 0°C., 760 MM. HG.	METABOLISM: Calories per 24 hours, Total	METABOLISM: Calories per 24 hours, Per kgm.	METABOLISM: Calories per hour, Total	METABOLISM: Calories per hour, Per sq. meter body surface	DIFFERENCE FROM CURVES OF: Harris Benedict (1919)	DIFFERENCE FROM CURVES OF: Du Bois Boothby Sandiford (1929)*
		yrs.	*cm.*	*kgm.*	*sq. meter*	*cc.*					*per cent*	*per cent*
75–79	LXXIV	75	173.5	77.4	1.91	232	1612	20.8	67.3	35.2		+2.8
	LXXV	75	164.5	67.8	1.72	205	1424	21.0	59.3	34.5		+0.9
	LXXVI	76	174.5	65.5	1.78	202	1403	21.4	58.5	32.9		−4.0
	LXXVII	77	167.0	69.6	1.77	204	1417	20.4	59.2	33.4		−2.4
	LXXVIII	77	161.0	65.0	1.68	199	1383	21.3	57.7	34.3		+0.3
	LXXIX	77	166.0	73.8	1.82	217	1508	20.4	62.9	34.6		+2.9
	LXXX	78	163.0	63.5	1.68	211	1466	23.1	61.1	36.4		+6.0)
			Average		1.766						Average	+0.9
80–84	LXXXI	80	171.0	64.0	1.74	170	1181	18.5	49.2	28.3		
	LXXXII	80	167.0	67.2	1.74	199	1383	20.6	57.6	33.1		
	LXXXIII	80	161.0	43.6	1.42	159	1105	25.3	45.9	32.3		
	LXXXIV	80	176.0	64.0	1.78	210	1459	22.8	60.8	33.6		
	LXXXV	81	165.5	72.6	1.79	237	1647	22.7	68.6	38.3		
	LXXXVI	81	159.5	65.1	1.66	190	1320	20.3	55.0	33.1		
	LXXXVII	82	184.0	76.8	1.98	261	1813	23.6	75.7	38.2		
	LXXXVIII	82	166.0	71.8	1.79	223	1549	21.6	64.5	36.0		
	LXXXIX	82	160.0	52.8	1.53	146	1015	19.2	42.3	27.6		
	XC	82	172.5	51.2	1.60	198	1376	26.9	57.3	35.8		
	XCI	82	153.5	45.0	1.38	158	1098	24.4	45.7	33.1		
	XCII	83	169.0	80.9	1.91	230	1598	19.8	66.6	34.9		
	XCIII	84	180.0	72.6	1.90	226	1570	21.6	65.4	34.4		
	XCIV	84	163.0	57.7	1.61	186	1292	22.4	53.9	33.5		
			Average		1.702							
85–89	XCV	85	168.5	56.2	1.63	177	1230	21.9	51.2	31.4		
	XCVI	85	162.5	53.8	1.55	203	1410	26.2	58.9	38.0		
	XCVII	85	168.0	62.8	1.71	190	1320	21.1	55.0	32.2		
	XCVIII	85	175.5	55.0	1.67	195	1355	24.6	56.4	33.8		
	XCIX	89	160.0	55.6	1.68	194	1348	24.2	56.3	33.5		
	C	89	163.0	58.4	1.61	176	1223	20.9	51.0	31.6		
			Average		1.642							
90–101	CI	91	179.0	72.6	1.90	198	1376	19.0	57.2	30.1		
	CII	91	167.0	61.6	1.68	177	1230	20.0	51.2	30.5		
	CIII	101	166.0	45.8	1.47	146	1014	22.1	42.2	28.7		
			Average		1.683							

† Age as of last birthday.

* The data in this column result from extrapolation of the curve of Du Bois modified by Boothby and Sandiford (1929).

straight line if the measurement is taken of the entire population between ages 40 and 89. The progressive decline is expressed by a regression curve which is derived from the use of the equation:[3]

$$\text{Calories} = M_C + r_{AC}\frac{\sqrt{\Sigma C^2 - (\Sigma C)(M_C)}}{\sqrt{\Sigma A^2 - (\Sigma A)(M_A)}}(A - M_A)$$

in which C = calories per square meter per hour; A = age in years; M = mean. Since r (the coefficient of correlation) is negative the solution of this equation is:

$$\bar{C} = 39.138 - 0.0678 \times A^{4}$$

When $A = 40, \bar{C} = 36.426$; and
$A = 89, \bar{C} = 33.106$ (fig. 1).

For the 50 year period the regression is accordingly 3.32 calories or 9.1 per cent. For each of the five decades beginning with age 40 it is 0.664

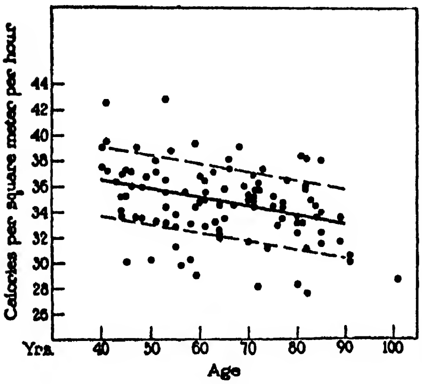

Fig. 1

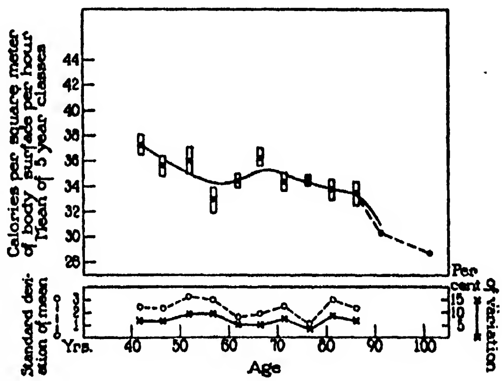

Fig. 2

Fig. 1. Basal metabolism in normal men over 40 years of age.

● individual observation.

——— regression of metabolism on age of 100 men, aged 40 to 89 years, twenty in each decade, with standard error of estimate - - - - - -.

Fig. 2. Basal metabolism in normal men over 40 years of age grouped according to half decades of age.

Hollow rectangles with solid dots represent the mean of 5 year classes, ± standard error of mean of age and of metabolism.

——— curve smoothed according to $\dfrac{N^a a + 2N^b b + N^c c.}{N^a + 2N^b + N^c}$

(Allowance for number of observations in each group.)

[3] For the methods of statistical analysis employed in this and subsequent papers see Fisher (11), Wallace and Snedecor (12), and Ezekiel (13).

[4] If, instead of a uniform distribution of 20 men per decade, the whole experience ranging from 40 to 101 years is taken, $\bar{C} = 39.481 - 0.0744 \times A$. In this case the curve is slightly steeper (fig. 4).

calories. Since the coefficient of correlation of metabolism and age is -0.3328 ± 0.0889 a highly significant negative correlation appears to exist (table 3).

c. Though the curve just given represents the course of events as an expression of the trend between ages 40 and 89, the facts actually require

TABLE 3

Summary of results of the statistical analysis of the basal metabolism, based on the data in table 2

AGE		NUMBER OF SUBJECTS	BASAL METABOLISM, CALORIES PER SQUARE METER PER HOUR					COEFFICIENT OF VARIATION $\frac{100\sigma_C}{M_C}$	COEFFICIENT OF CORRELATION r_{AC}	STANDARD ERROR OF CORRELATION (r_{AC}) σ_r	VALUE OF t	LEVEL OF SIGNIFICANCE FISHER'S TABLES
Group	Mean		Minimum	Maximum	Mean M_C	Standard deviation σ_C	Standard error of mean σ_{M_C}					
yrs.	*yrs.*							*per cent*				
40–44	42.20	10	33.7	42.6	37.3	2.55	0.848	6.8	−0.7305	±0.1554	4.7	
45–49	46.60	10	30.2	39.2	35.6	2.40	0.800	6.8	+0.1959	±0.3205	0.6	
50–54	52.00	10	50.3	42.9	36.1	3.35	1.115	9.3	+0.5837	±0.2198	2.7	
55–59	57.10	10	29.0	39.4	32.9	2.95	0.984	9.0	+0.2190	±0.3173	0.7	
60–64	62.31	13	31.9	37.2	34.5	1.73	0.503	5.0	−0.4994	±0.2167	2.3	
65–69	66.67	7	33.4	39.1	36.3	1.86	0.761	5.1	−0.3675	±0.3532	1.0	
70–74	71.46	13	28.1	37.3	34.4	2.50	0.723	7.3	−0.1239	±0.2842	0.4	
75–79	76.43	7	32.9	36.4	34.5	1.06	0.433	3.1	+0.2562	±0.3815	0.7	
80–84	81.64	14	27.6	38.3	33.7	2.96	0.820	8.6	+0.2395	±0.2614	0.9	
85–89	86.33	6	31.4	38.0	33.4	2.24	1.002	6.7	−0.2747	±0.4204	0.7	
90–94	91.00	2	30.1	30.5	30.3	0.20	0.200	0.7	0	0	0	
101		1	28.7									
40–49	44.40	20	30.2	42.6	36.4	2.62	0.601	7.2	−0.4439	±0.1842	2.4	
50–59	54.55	20	29.0	42.9	34.5	3.53	0.809	10.2	−0.2181	±0.2185	1.0	
60–69	63.80	20	31.9	39.1	35.1	1.99	0.451	5.7	+0.2501	±0.2151	1.2	
70–79	73.20	20	28.1	37.3	34.4	2.11	0.485	6.1	−0.0110	±0.2294	0.005	
80–89	83.05	20	27.6	38.0	33.6	2.77	0.634	8.2	+0.0018	±0.2294	0.0008	
90–101	94.33	3	28.7	30.5	29.8	0.77	0.546	2.6	−0.7336	±0.3267	2.2	
40–64	52.63	53	29.0	42.6	35.2	2.99	0.410	8.5	−0.3403	±0.1215	2.8	Significant
65–89	76.40	47	27.6	39.1	34.4	2.54	0.371	7.4	−0.3427	±0.1287	2.7	Significant
65–101	77.48	50	27.6	39.1	34.1	2.71	0.383	7.9	−0.4439	±0.1136	3.9	Highly significant
40–89	63.80	100	27.6	42.6	34.8	2.82	0.282	8.1	−0.3328	±0.0889	3.7	Highly significant
40–101	64 69	103	27.6	42.6	34.7	2.91	0.286	8.4	−0.3735	±0.0848	4.4	Highly significant

a more complex presentation if the mean levels at succeeding decades or half-decades are taken into account. It has been necessary to learn, therefore, what the average basal rate is in each successive age group. The mean values have accordingly been ascertained decade by decade and at half-decade intervals (table 3).

The decline is less orderly if the averages obtained in five-year groups are plotted (fig. 2), more orderly if those of ten-year periods (fig. 3) are used. At 40 to 44 years, the mean value was 37.28 calories per square meter per hour, and at 45 to 49, 50 to 54, 65 to 69 years it was approximately 36 calories, but at 55 to 59 years an unexpected irregularity brought about a fall in the curve to 32.9 calories. After age 70 the mean values were found progressively to fall.

If, instead of the means at five-year intervals, those of whole decades are plotted, the resulting curve takes on a more orderly or consistent ap-

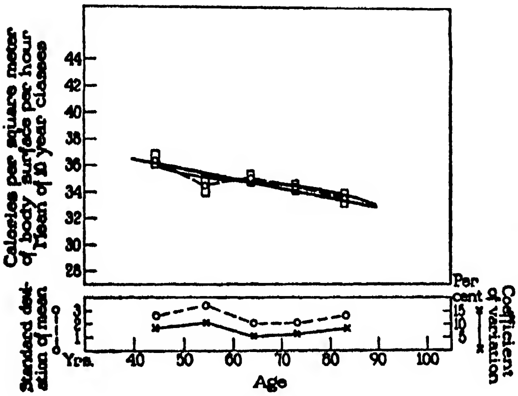

Fig. 3

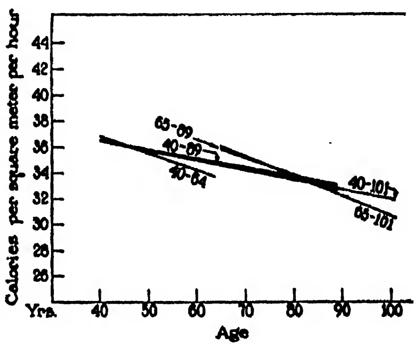

Fig. 4

Fig. 3. Basal metabolism in normal men over 40 years of age grouped according to decades of age.

Hollow rectangles with open dots represent the mean of 10 year classes, twenty men in each class, ± standard error of mean.

——— curve of 10 year means smoothed according to $\frac{a + 2b + c.}{4}$

▬▬▬ curve of regression, 40 to 89 years.

Fig. 4. Basal metabolism in normal men over 40 years of age. Curves of regression of various age groups.

pearance (fig. 3). There is then a decline which is gradual through the 6th, 7th and 8th decades.

It is improbable that the curve as now presented is definitive.[5] It seems necessary to believe a smooth curve is descriptive of the course of events.

From what is already known of changes in the rate of metabolism it is not unexpected that after age 40 the slope of the curve which describes them undergoes inflection. It is only in the earliest years that the level rises sharply. Afterward it exhibits declines, at various rates, or steadiness. The declines are marked in childhood (ages 2 to 12) and again after

[5] This situation is not unique. See Harris and Benedict (7), pp. 120–122, and Du Bois (4) pp. 126–128.

adolescence (4, 10, 14, 15, 16). During adolescence the rate of decline may be the same (14, 15, 16), or more probably arrested (1, 4, 17, 18, 19). The period of greatest steadiness is found between 20 years and 40 years though here also there is a slight decline. A decline then sets in again about the exact nature of which there is doubt. As the result of this study no more can be definitely inferred than that the slope of the curve of regression undergoes one or more inflections between its end points

TABLE 4

*Basal metabolism in normal adult men by half-decades of age between 40 and 104 years**

1	2	3	4	5	6
AGE GROUP	BASAL METABOLISM, CALORIES PER SQUARE METER PER HOUR		CHANCES OF BEING NORMAL WHEN VARIATION FROM MEAN IS MORE THAN		
	Mean	Standard error of estimate S	1S = 1 in 3	2S = 1 in 20	2½S = 1 in 100
yrs.			*per cent*	*per cent*	*per cent*
40–44	36.4	2.69	7.5	15.0	18.5
45–49	36.1	2.69	7.5	15.0	19.0
50–54	35.7	2.69	7.5	15.0	19.0
55–59	35.4	2.69	7.5	15.0	19.0
60–64	35.0	2.69	7.5	15.5	19.5
65–69	34.7	2.69	8.0	15.5	19.5
70–74	34.4	2.69	8.0	15.5	19.5
75–79	34.0	2.69	8.0	16.0	20.0
80–84	33.7	2.69	8.0	16.0	20.0
85–89	33.3	2.69	8.0	16.0	20.0
90–94	32.6	2.73	8.5	17.0	21.0
95–99	32.3	2.73	8.5	17.0	21.0
100–104	31.9	2.73	8.5	17.0	21.0

* The results from 40 to 89 are calculated with the use of the equation:

$$\text{Calories per square meter per hour} = 39.138 - 0.0678 \times \text{Age};$$

and from 90 to 104 years with the use of the equation:

$$\text{Calories} = 39.481 - 0.0744 \times \text{Age}$$

The chances of being normal are given when the variation from the average value (column 2) is greater than one standard error of estimate (1 chance in 3, column 4) 2 times the standard error of estimate (1 chance in 20, column 5); and 2½ times the standard error of estimate (1 chance in 100, column 6).

(40 and 89 years) (fig. 4). The accumulation of more measurements should make it possible to define with reasonable accuracy precisely what the course of the curve actually is in the long period of decline from 40 to 90. There are of course a number of possibilities: *1*, that the plateau of middle life is prolonged; *2*, that it descends in a fashion illustrated by an S-shaped curve; *3*, that after a period of 15 years, until 55 years, it remains level, thence to descend in a manner illustrated by a parabolic curve.

The values given in the equation in paragraph *b* are recapitulated in

table 4. The standard error of estimate for the men, 40 to 89 years of age, is 2.69 calories per square meter per hour. The chances of being average are given in table 4 and figure 6.

Discussion. The state of knowledge of the changes in the rate of metabolism with age, at least in the later decades, is not yet in a satisfactory situation as a study of all the measurements over 40 years of age now available indicates (table 1).

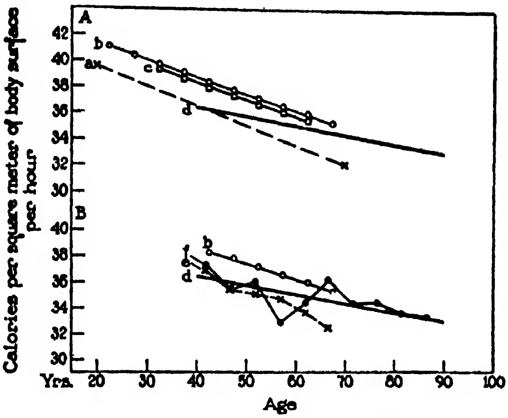

Fig. 5

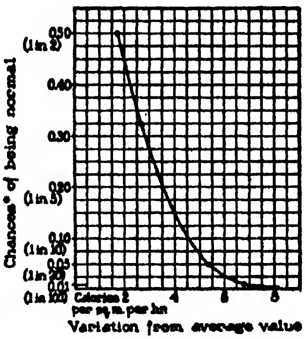

Fig. 6

Fig. 5. *A*. Comparison of the regression curves obtained from the present study with those of Harris and Benedict (1919) and of Du Bois as modified by Boothby and Sandiford (1929), and Boothby, Berkson, and Dunn (1936).

a, regression by prediction equation, Harris and Benedict (1919).

b, regression by 5 year means, Boothby and Sandiford (1929).

c, regression by 5 year means, Boothby, Berkson, and Dunn (1936).

d, regression by prediction equation, this study.

B. Curves based on the present study of 5 year groups.

b, as in *A*.

d, as in *A*.

e, regression by 5 year means for subjects of this study calculated from tables by Harris and Benedict (1919).

f, regression by 5 year means, this study.

Fig. 6. A curve showing the probability that the observed basal metabolism is normal expressed as variations from the average value in men over 40 years of age.

* The phrase used for this expression by Ezekiel (1930) is the "probability of specified departure" from the average value.

Note: When in an individual the basal rate differs 6.7 calories from the average the chances are only 1:100 that this measurement is normal.

When the straight curve resulting from this analysis is compared with that of Du Bois as modified by Boothby and Sandiford (16), and by Boothby, Berkson, and Dunn (20) and with that of Harris and Benedict (7), its slope is less steep than that of the other two,—0.7 calorie per square meter per hour against theirs of 1.3, and lies about mid-way between

the two (fig. 5).[6] The divergence of the Harris-Benedict curve is marked in the later years. The cause of this is not quite certain, but it may depend on the fact that their sample contained predominantly young subjects. There were only eleven individuals over age 40 and none older than 64. There exists also the factor of greater variation in the metabolism of elderly subjects, noted again by Du Bois (4) and Benedict (21) after further recent experience.

Variations such as these recall the statement of Gephart and Du Bois (22)[7] that "the selection of a proper normal base-line is a matter of great difficulty" and the one of Harris and Benedict (7)[8] that "the difficulties associated with variations in basal metabolism suggest that the difficulties of the selection of proper controls have been underestimated rather than overestimated in the past."

CONCLUSIONS

1. The basal metabolic rates have been studied in 100 normal men, 20 in each decade from 40 to 89 years. In addition measurements were obtained of 2 men at 91 years, and one at 101 years. It was found that the rate of metabolism falls between 40 and 89 and that the fall can be expressed by the equation: The calories lost per square meter per hour $= 39.138 - 0.0678 \times$ age. The negative correlation is -0.3328 ± 0.0889 and is highly significant. In each decade, the regression is 0.664 calorie per square meter per hour.

2. The mean values in succeeding decades suggest that the rate of decline of the curve is not straight but may be arrested between 50 years and 79 years. The curve obtained is not smooth; were more than 20 individuals examined in each decade it might become so. That variation in each age group exists has been learned again in this investigation. In any 5 or 10 year group the maximum deviations from the respective means were -18.3 and $+24.4$ per cent. The standard deviation, the standard error of the mean and the coefficient of correlation have therefore been calculated in 5, 10 and 25 year groups.

For the entire series (40 to 101) the mean is 34.667 calories per square meter per hour; the standard deviation is 2.906 (8.4 per cent of the mean) and the standard error 0.286 calories. For the evenly distributed group (40 to 89) the mean is 34.814, the standard deviation 2.813 (8.1 per cent

[6] Du Bois (4), 3rd ed. revised, 1936, p. 171. "We may conclude that there is a great deal of variation in old people, probably depending upon the degree of senility, and that if the general slope of the lines of the standards are continued into old age, the Harris-Benedict level is somewhat too low and the Aub and Du Bois and Boothby a little too high."

[7] P. 857.

[8] P. 224.

of the mean), the standard error of the mean 0.282, and the standard error of estimate 2.69 calories per square meter per hour.

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CHANGES WITH AGE IN THE CARDIAC OUTPUT IN ADULT MEN[1]

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The maintenance of a usual volume of output[2] of blood per minute from the heart is among the important functions of the circulation. This volume in normal young men and women under so-called basal conditions has been found, in recent years, to be 2.2 ±0.3 liters per square meter of body surface (1). In the same individual under the same conditions the amount is the same from day to day. It is approximately the same, furthermore, in individuals of the same sex and size of body. But whether it remains the same, irrespective of age, is still unknown. A few measurements have been made in children (1b), in whom the index, in keeping with their higher metabolic rate, is relatively higher than in adults. From other publications may be collected the results of a few measurements, made for other purposes, in individuals over forty years of age. But knowledge of the variation of the cardiac index with age is, as Grollman (1b) has pointed out, deficient.

This communication reports the changes in the volume of the index which takes place under basal conditions, in normal men over forty years of age. The subjects were one hundred men from forty to eighty-nine years, twenty in each of the five decades; there were in addition two men of ninety-one, and one of one hundred and one years. They were the same men who were described in the preceding report (2). They were studied under uniform and basal conditions in the manner there described.

METHOD. For measuring the cardiac output the acetylene rebreathing procedure was employed. Accuracy in technique in the use of the method requires: *1*, that complete diffusion of the gas mixture in the lung-bag system be attained before the first sample of gas is taken for analysis; and *2*, that later samples be taken before blood containing acetylene has returned to the lungs. In normal young adults, six deep respirations in 15 seconds have been found sufficient to attain diffusion, and a lapse of

[1] This paper is no. 18 of the series on Physiological Ontogeny.

[2] The phrase "cardiac output" when used in these papers means the amount of blood expelled by the heart in a minute. If the calculation is made in terms of the surface area of the body, the phrase "cardiac index" (*CI*) is used.

eight or ten seconds thereafter sufficient, before return of the blood to the lungs (3, 4). To be certain these conditions are fulfilled, three samples are taken in succession; if the results derived from the first and second samples and from the second and third samples are reasonably similar, the test is regarded as having been correctly made (5) (fig. 1). In this study three or four samples were always taken; those tests were considered satisfactory in which the difference in the result from samples A and B, and that from B and C was less than 12 per cent of the average of A and C. Closer agreement was not found possible. Such checks are especially important in elderly persons. In the first 45 normal subjects the mean

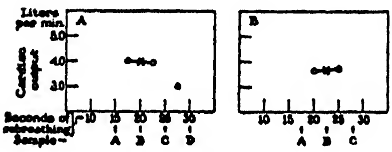
Fig. 1

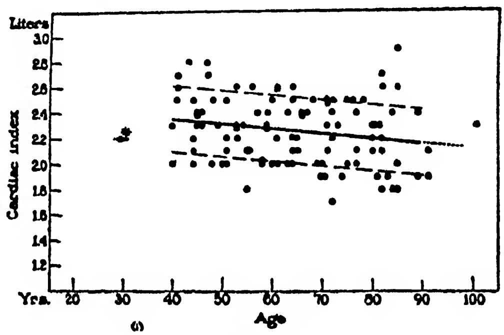
Fig. 2

Fig. 1. The method of control of tests of gas samples for measuring cardiac output.

A. Subject II, 40 years; 4 samples were taken at 15(A), 20(B), 25(C), 30(D) seconds of rebreathing.

B. Subject XCII, 83 years; 3 samples were taken at 17(A), 22(B), 27(C) seconds of rebreathing.

Fig. 2. The cardiac index in normal men over 40 years of age.

● individual observations.

——— regression of cardiac index on age, for 100 men, aged 40 to 89 years, twenty in each decade, extended ········· to include 3 men over 89 years, with curves - - - - - of standard error of estimate.

*Average in normal men aged 21 to 35 years, Grollman (1).

deviation of the results of samples A and B or B and C from the average of A and C, was 4.7 per cent.

In the early experiments four samples were taken in succession. In a few subjects, measurements were made on succeeding days; no significant deviations appeared.

Results are reported in several ways:[3] *a.* The mean value of the cardiac index in one hundred men from forty to eighty-nine years, separated in decades or half decades, ranged from 2.40 to 2.17 liters; in the series of one hundred men taken as a whole, it was 2.26 liters (table 1, fig. 2). These values resemble the average 2.2 liters found in young men of twenty to

[3] References to the statistical methods employed are given in preceding paper (2).

TABLE 1

Measurements of certain circulatory phenomena: data for the calculation of the cardiac index

GROUP	SUBJECT NO.	PULSE RATE PER MINUTE	OXYGEN CONSUMPTION PER MINUTE (WITHOUT CORRECTION FOR BAROMETER AND TEMPERATURE)	ARTERIO-VENOUS OXYGEN DIFFERENCE PER LITER OF BLOOD	CARDIAC OUTPUT PER MINUTE	CARDIAC INDEX (OUTPUT PER SQUARE METER OF BODY SURFACE PER MINUTE)	SYSTOLIC OUTPUT	SYSTOLIC OUTPUT PER SQUARE METER OF BODY SURFACE	BLOOD PRESSURE, MILLIMETERS Hg: Systolic, Right arm	Systolic, Left arm	Diastolic, Right arm	Diastolic, Left arm	X-RAY OF HEART: Transverse diameter	Cardiac area
			cc.	*cc.*	*liters*	*liters*	*cc.*	*cc.*					*cm.*	*sq. cm.*
40–44	I	56	283	72.6	3.9	2.0	70	35	118	110	78	72	14.80	107.2
	II	70	253	64.2	3.9	2.3	56	33	110	100	76	70	13.91	129.2
	III	56	286	68.6	4.8	2.7	86	48	104	96	62	54	14.31	115.5
	IV	62	284	57.6	4.9	2.6	79	42	128	118	80	76	13.30	122.5
	V	66	265	58.2	4.6	2.5	71	38	136	120	0	80	12.10	84.9
	VI	60	259	50.6	5.1	2.8	85	45	115	115	70	64	13.50	128.7
	VII	58	233	58.3	4.0	2.5	69	43	100	90	70	70	12.80	98.7
	VIII	64	211	61.7	3.4	2.2	53	33	112	114	70	70	12.39	107.5
	IX	56	274	65.8	4.2	2.0	75	35	126	110	92	80	15.80	109.2
	X	66	242	62.7	3.9	2.1	59	31	110	100	76	70	14.0	111.9
45–49	XI	58	235	58.4	4.0	2.3	69	39	124	140	84	86	12.38	100.1
	XII	68	265	57.7	4.6	2.4	68	36	130	120	82	86	11.90	94.1
	XIII	54	221	49.4	4.5	2.3	83	43	136	106	82	80	15.30	107.5
	XIV	66	243	57.3	4.2	2.5	64	38	122	114	80	70	14.30	104.2
	XV	56	263	60.3	4.4	2.3	79	42	106	106	76	76	15.10	123.1
	XVI	70	245	47.7	5.1	2.7	73	39	110	90	62	56	14.10	108.7
	XVII	60	295	54.6	5.4	2.8	90	46	116	100	70	68	15.40	117.9
	XVIII	54	214	65.9	3.2	2.0	59	37	124	116	86	82	11.99	108.9
	XIX	62	257	55.2	4.7	2.4	76	39	120	106	80	72	14.95	88.7
	XX	60	225	60.5	3.7	2.3	62	37	118	120	80	78	11.80	88.4
50–54	XXI	62	231	58.6	3.9	2.0	63	32	104	108	62	72	13.80	99.6
	XXII	64	270	69.4	3.9	2.1	61	33	154	150	88	90	12.95	91.9
	XXIII	55	238	56.9	4.2	2.5	76	46	110	110	60	60	11.41	113.5
	XXIV	66	218	58.0	3.8	2.2	58	34	148	148	90	90	14.80	122.9
	XXV	58	218	63.6	3.4	2.0	59	34	120	115	80	60	13.80	106.9
	XXVI	82	248	58.6	4.1	2.2	50	28	116	116	74	76	14.05	96.9
	XXVII	57	257	59.8	4.3	2.3	75	39	130	120	85	80	14.50	95.7
	XXVIII	60	214	55.3	3.9	2.6	65	43	110	110	85	80	10.99	99.2
	XXIX	54	311	71.2	4.4	2.3	81	42	125	112	70	70	15.00	123.2
	XXX	60	253	65.5	3.9	2.3	65	39	110	100	74	64	11.65	115.1

TABLE 1—*Continued*

GROUP	SUBJECT NO.	PULSE RATE PER MINUTE	OXYGEN CONSUMPTION PER MINUTE (WITHOUT CORRECTION FOR BAROMETER AND TEMPERATURE)	ARTERIO-VENOUS OXYGEN DIFFERENCE PER LITER OF BLOOD	CARDIAC OUTPUT PER MINUTE	CARDIAC INDEX (OUTPUT PER SQUARE METER OF BODY SURFACE PER MINUTE)	SYSTOLIC OUTPUT	SYSTOLIC OUTPUT PER SQUARE METER OF BODY SURFACE	BLOOD PRESSURE, MILLIMETERS Hg: Systolic, Right arm	Systolic, Left arm	Diastolic, Right arm	Diastolic, Left arm	X-RAY OF HEART: Transverse diameter	X-RAY OF HEART: Cardiac area
			cc.	*cc.*	*liters*	*liters*	*cc.*	*cc.*					*cm.*	*sq. cm.*
55–59	XXXI	60	232	65.4	3.5	1.8	58	30	160	130	108	90	15.00	118.2
	XXXII	57	224	62.4	3.6	2.1	63	36	120	116	80	76	12.81	107.8
	XXXIII	63	252	59.5	4.2	2.1	67	34	108	102	66	66	14.50	102.5
	XXXIV	72	200	45.2	4.4	2.6	61	35	104	100	70	72	12.60	113.9
	XXXV	48	251	56.4	4.4	2.4	92	51	90	100	68	68	14.10	101.3
	XXXVI	64	224	62.2	3.6	2.0	56	32	140	118	78	70	12.39	105.2
	XXXVII	56	216	58.9	3.7	2.0	66	36	124	110	96	74	14.00	89.9
	XXXVIII	48	295	66.8	4.4	2.3	92	48	126	116	86	86	15.60	105.2
	XXXIX	57	204	47.7	4.3	2.3	75	41	130	128	82	80	13.70	96.9
	XL	74	275	56.2	4.9	2.4	66	32	156	152	94	90	15.60	83.7
60–64	XLI	50	245	55.6	4.4	2.5	88	51	106	106	70	70	13.40	106.5
	XLII	62	224	63.2	3.5	2.0	56	33	110	100	70	64	12.51	109.2
	XLIII	72	232	50.7	4.6	2.6	64	37	140	128	90	78	13.70	93.5
	XLIV	78	255	54.3	4.7	2.6	60	33	170	150	90	86	14.40	95.2
	XLV	58	237	61.3	3.9	2.2	67	38	140	130	80	86	14.75	129.2
	XLVI	64	263	62.3	4.2	2.0	66	32	114	102	76	66	16.25	125.5
	XLVII	68	290	60.2	4.8	2.4	71	36	120	104	70	60	15.01	96.2
	XLVIII	52	229	66.0	3.5	2.0	67	37	134	126	82	84	14.80	111.9
	XLIX	54	244	60.3	4.0	2.2	74	40	116	110	80	70	15.20	102.5
	L	60	209	68.2	3.4	2.1	57	35	100	94	66	62	12.05	100.3
	LI	54	209	51.7	4.1	2.6	76	48	102	95	58	50	12.05	100.9
	LII	58	226	61.7	3.7	2.0	64	35	132	116	76	70	15.30	108.1
	LIII	68	212	51.5	4.3	2.5	63	36	128	120	70	78	12.50	118.1
65–69	LIV	52	272	65.2	4.2	2.2	81	42	140	136	80	80	13.78	106.5
	LV	64	213	61.2	3.5	2.1	55	33	146	118	76	60	14.65	116.2
	LVI	52	255	62.6	4.1	2.4	79	45	140	118	88	78	13.90	120.7
	LVII	63	240	59.3	4.1	2.4	65	38	110	120	70	80	12.15	85.4
	LVIII	60	274	54.7	5.0	2.4	83	40	140	140	80	80	15.10	118.8
	LIX	63	238	59.3	4.0	2.5	63	40	140	130	80	72	11.00	98.9
	LX	50	220	68.5	3.2	2.0	64	39	124	126	54	54	14.95	118.6

TABLE 1—*Continued*

GROUP	SUBJECT NO.	PULSE RATE PER MINUTE	OXYGEN CONSUMPTION PER MINUTE (WITHOUT CORRECTION FOR BAROMETER AND TEMPERATURE)	ARTERIO-VENOUS OXYGEN DIFFERENCE PER LITER OF BLOOD	CARDIAC OUTPUT PER MINUTE	CARDIAC INDEX (OUTPUT PER SQUARE METER OF BODY SURFACE PER MINUTE)	SYSTOLIC OUTPUT	SYSTOLIC OUTPUT PER SQUARE METER OF BODY SURFACE	BLOOD PRESSURE, MILLIMETERS Hg: Systolic, Right arm	Systolic, Left arm	Diastolic, Right arm	Diastolic, Left arm	X-RAY OF HEART: Transverse diameter	X-RAY OF HEART: Cardiac area
			cc.	cc.	liters	liters	cc.	cc.					cm.	sq. cm.
70–74	LXI	52	234	69.6	3.4	1.9	65	37	120	118	68	64	14.10	87.5
	LXII	58	275	72.5	3.8	1.9	66	34	128	136	80	80	15.90	112.4
	LXIII	66	183	60.4	3.0	2.0	45	29	150	136	78	60	11.90	65.1
	LXIV	56	271	66.1	4.1	2.1	73	38	176	130	80	60	14.95	99.8
	LXV	66	218	54.8	4.0	2.5	61	37	160	150	80	88	12.50	81.5
	LXVI	60	209	56.4	3.7	2.3	62	39	114	110	74	70	14.00	98.3
	LXVII	64	218	53.6	4.1	2.5	64	39	138	130	76	76	12.85	104.3
	LXVIII	62	267	63.4	4.2	2.2	68	35	130	144	70	70	14.39	95.2
	LXIX	47	213	57.0	3.7	2.4	79	52	136	140	68	70	12.60	104.9
	LXX	56	193	61.9	3.1	1.7	55	30	110	105	60	55	14.45	100.6
	LXXI	56	225	54.4	4.1	2.5	73	45	126	142	64	72	12.80	83.2
	LXXII	66	273	60.8	4.5	2.3	68	35	180	150	100	86	15.65	122.9
	LXXIII	54	211	62.6	3.4	1.9	63	35	170	155	90	90	13.91	120.4
75–79	LXXIV	56	255	53.9	4.7	2.5	84	44	176	160	96	82	14.30	98.5
	LXXV	54	230	68.1	3.4	2.0	63	37	150	140	90	75	15.20	115.4
	LXXVI	54	227	51.9	4.4	2.5	81	46	140	130	64	64	12.60	82.9
	LXXVII	56	224	60.7	3.7	2.1	66	37	120	130	80	72	13.60	107.2
	LXXVIII	66	221	64.5	3.4	2.0	52	31	150	150	90	80	14.79	100.5
	LXXIX	50	243	54.6	4.4	2.4	88	48	168	126	80	64	14.20	106.4
	LXXX	76	234	55.3	4.2	2.5	55	33	170	170	98	100	13.40	101.5
80–84	LXXXI	50	190	48.9	3.9	2.3	78	45	150	140	80	80	15.20	115.4
	LXXXII	60	218	56.3	3.9	2.2	65	37	180	160	80	82	14.99	103.2
	LXXXIII	62	176	52.8	3.3	2.3	53	37	150	148	74	78	12.20	113.9
	LXXXIV	57	214	63.1	3.7	2.1	65	37	110	112	50	46	12.40	99.1
	LXXXV	50	259	61.1	4.2	2.3	84	47	180	180	70	70	17.90	156.6
	LXXXVI	72	207	66.4	3.1	1.9	43	26	150	150	76	76	13.70	90.5
	LXXXVII	72	296	56.7	5.2	2.6	72	36	180	180	90	90	16.40	145.6
	LXXXVIII	74	249	65.4	3.8	2.1	51	28	128	116	70	70	15.70	97.1
	LXXXIX	54	162	58.1	2.8	1.8	52	34	130	120	60	60	13.70	118.7
	XC	62	221	50.8	4.3	2.7	69	43	178	184	84	88	14.10	101.4
	XCI	54	177	56.4	3.1	2.2	57	41	150	136	80	70	14.10	110.5
	XCII	74	259	70.5	3.7	1.9	50	26	170	144	92	80	20.00	157.2
	XCIII	78	254	55.1	4.6	2.4	59	31	140	130	80	80	13.80	110.9
	XCIV	60	203	70.8	2.9	1.8	48	30	160	150	80	76	14.90	97.5

TABLE 1—*Concluded*

GROUP	SUBJECT NO.	PULSE RATE PER MINUTE	OXYGEN CONSUMPTION PER MINUTE (WITHOUT CORRECTION FOR BAROMETER AND TEMPERATURE)	ARTERIO-VENOUS OXYGEN DIFFERENCE PER LITER OF BLOOD	CARDIAC OUTPUT PER MINUTE	CARDIAC INDEX (OUTPUT PER SQUARE METER OF BODY SURFACE PER MINUTE)	SYSTOLIC OUTPUT	SYSTOLIC OUTPUT PER SQUARE METER OF BODY SURFACE	BLOOD PRESSURE, MILLIMETERS Hg: Systolic, Right arm	Systolic, Left arm	Diastolic, Right arm	Diastolic, Left arm	X-RAY OF HEART: Transverse diameter	Cardiac area
			cc.	*cc.*	*liters*	*liters*	*cc.*	*cc.*					*cm.*	*sq. cm.*
85-89	XCV	62	202	63.0	3.2	2.0	52	38	130	132	70	72	12.80	109.6
	XCVI	76	228	50.8	4.5	2.9	59	38	140	140	78	78	11.79	82.2
	XCVII	60	209	70.8	3.0	1.8	50	30	195	195	85	85	14.00	100.9
	XCVIII	68	214	49.0	4.4	2.6	65	39	130	124	70	74	11.60	108.7
	XCIX	65	222	53.9	4.1	2.4	63	38	188	190	76	80	12.75	76.3
	C	48	200	64.2	3.1	1.9	65	40	164	140	86	76	13.30	99.9
90-101	CI	60	225	59.9	3.8	1.9	63	33	124	110	70	68	14.90	104.4
	CII	58	200	56.4	3.6	2.1	62	37	170	174	80	82	13.80	101.8
	CIII	56	164	48.8	3.4	2.3	60	41	136	130	64	58	11.70	69.6

thirty years (1). The mean index is slightly higher in this group in the years from 40 to 69 years than was observed by Grollman in young men. After 70 years it is the same.

The range of variation in each age group was approximately the same, except for the wider range in the ninth decade (figs. 3, 4, table 2). The smallest index observed in the entire series was 1.7; the greatest, 2.9 liters. In the entire scatter, the range is greater than ±0.3 liter, given by Grollman; but Grollman's results were derived from a group of medical students unusually homogeneous as to age and social station. The better trained and the more uniform the group, the more likely is it, natually, to yield uniform values. The range of the scatter in this series may have been due to the random selection of the sample; in any case it was not great. In the three men, ninety-one and one hundred and one years of age, the cardiac indices were 1.9, 2.1 and 2.3 liters.

The standard deviations in the different age groups on the whole varied only moderately (table 2, figs. 3, 4). The largest in the 5-year classes was ±0.38 liter or ±16.6 per cent and occurred in the group aged eighty-five to eighty-nine years; otherwise, it varied from ±7.6 to ±12.2 per cent. For the one hundred men it was ±0.26 liter or ±11.5 per cent of the mean.

b. A curve drawn to represent the facts shows that there is a general trend according to which the cardiac index declines slightly with age when the mean values of the age groups are joined (figs. 3, 4). The decline

TABLE 2

Summary of results of the statistical analysis of the cardiac index, based on the data in table 1

AGE GROUP	NUMBER OF SUBJECTS	CARDIAC INDEX: Minimum	CARDIAC INDEX: Maximum	CARDIAC INDEX: Mean M_{CO}	CARDIAC INDEX: Standard deviation σ_{CO}	CARDIAC INDEX: Standard error of mean $\sigma_{M_{CO}}$	COEFFICIENT OF VARIATION $\frac{100\sigma_{CO}}{M_{CO}}$	COEFFICIENT OF CORRELATION $r_{A \cdot CO}$	STANDARD ERROR OF CORRELATION $(r_{A \cdot CO})$ σ_r	VALUE OF t	LEVEL OF SIGNIFICANCE FISHER'S TABLES
years		*liters*	*liters*	*liters*	*liter*	*liter*	*per cent*				
40–44	10	2.0	2.8	2.37	0.276	0.092	11.6				
45–49	10	2.0	2.8	2.40	0.214	0.071	8.9				
50–54	10	2.0	2.6	2.25	0.186	0.062	8.3				
55–59	10	1.8	2.6	2.20	0.228	0.076	10.4				
60–64	13	2.0	2.6	2.28	0.244	0.071	10.7				
65–69	7	2.0	2.5	2.29	0.173	0.071	7.6				
70–74	13	1.7	2.5	2.17	0.261	0.075	12.1				
75–79	7	2.0	2.5	2.29	0.223	0.091	9.8				
80–84	14	1.8	2.7	2.19	0.267	0.074	12.2				
85–89	6	1.8	2.9	2.27	0.377	0.169	16.6				
90–94	2	1.9	2.1	2.0	0.10	0.10	5.0				
101	1	2.3									
40–49	20	2.0	2.8	2.38	0.248	0.057	10.4				
50–59	20	1.8	2.6	2.22	0.197	0.045	8.9				
60–69	20	2.0	2.6	2.28	0.222	0.051	9.7				
70–79	20	1.7	2.5	2.21	0.255	0.058	11.5				
80–89	20	1.8	2.9	2.21	0.314	0.072	14.2				
90–101	3	1.9	2.3	2.10	0.163	0.116	7.8				
40–89	100	1.7	2.9	2.26	0.261	0.026	11.5	−0.1862	±0.0965	1.9	Not significant
40–101	103	1.7	2.9	2.26	0.261	0.026	11.6	−0.2039	±0.0944	2.2	Significant

appears progressive when the whole population is considered and is then presented by a straight line (fig. 2). If all the cases in the age span forty to eighty-nine years are treated together, the equation for the decline is:

$$\overline{CI} = 2.49 - 0.0035 \times A$$

$$A = \text{age in years}$$

When

$$A = 40, \text{ then } \overline{CI} = 2.35 \text{ liters;}$$
$$A = 90, \text{ then } \overline{CI} = 2.17 \text{ liters.}$$

The decline during the 50 year period is, accordingly, 0.18 liter or 7.3 per cent. For each decade the decline is accordingly 0.035 liter or 1.5 per cent.

The coefficient of correlation of age and index, −0.1862 ±0.0965,

indicates that the two follow an inverse relation; but it is not sufficiently high to be significant. If the results of the entire series, including the three men over eighty-nine years, are correlated with age, the coefficient, -0.2039 ± 0.0944, is significant. The regression equation then would be

$$\overline{CI} = 2.49 - 0.0036 \times A$$

When
$$A = 40,\ \overline{CI} = 2.35 \text{ liters};$$
$$A = 90,\ \overline{CI} = 2.17 \text{ liters};$$
$$A = 100,\ \overline{CI} = 2.13 \text{ liters}.$$

The amount and rate of decline is the same as in the curve of 100 men, aged forty to eighty-nine years.

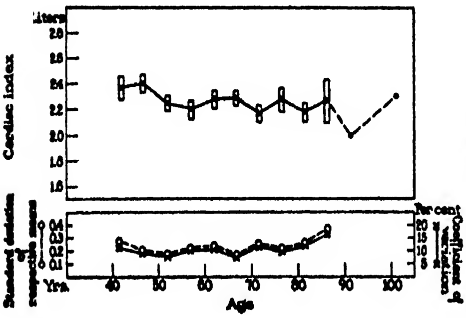

Fig. 3

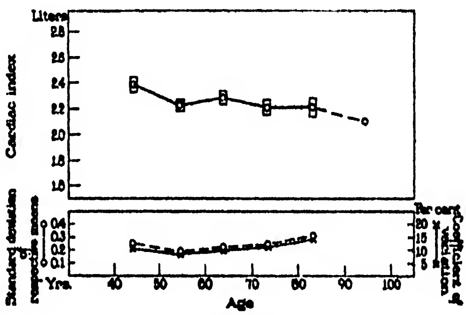

Fig. 4

Fig. 3. Cardiac index in normal men over 40 years of age, divided in 5 year groups. Hollow rectangles with solid dots represent the mean of 5 year classes, ± standard error of mean of age and of cardiac index.

Fig. 4. Cardiac index in normal men over 40 years of age, divided in 10 year groups.

Hollow rectangles with open dots represent the mean of 10 year classes, ± standard error of mean.

c. Though the general trend of the cardiac index is downward, the mean values if plotted at five or ten year periods indicate that inflections in the curve may exist (figs. 3, 4). The decline appears to be steeper in the forty to fifty-five year period. Then the rate of decline becomes arrested, the decline appearing less orderly when viewed in five year periods, more orderly in ten. The average index in the 20 subjects in the eighth decade and in the 3 subjects over 90 is the same, 2.21 liters. The changes from one to the next period are slight; accordingly the coefficients of correlation are not significant (table 2). The absence of a uniform slope throughout the period forty to one hundred and one suggests, though, that the measurement of more cases is desirable. Since the measurement of the cardiac index in Grollman's method depends on knowing the volume of oxygen consumed, there should be a relation between the decline in the rate of metabolism and a decline in the cardiac index.

The measurement of the volume of the cardiac index, according to the Fick principle, involves the relation of two potentially variable functions, the oxygen consumption or numerator, and the arterio-venous oxygen difference, or denominator, in an equation. The method of calculation reflects the conception that variation in the index follows directly, changes in oxygen consumption; but inversely, changes in the arterio-venous oxygen difference.[4] Since in young men the arterio-venous oxygen difference varies little, the index in them varies directly and quantitatively with oxygen consumption (7, 1a). Should the arterio-venous oxygen difference undergo change in men of older ages, however, the index would no longer be expected to run parallel with oxygen consumption. In the 100 men representing the 50 year period from 40 to 89 years, who form the basis of this study, the arterio-venous oxygen difference declined 3.2

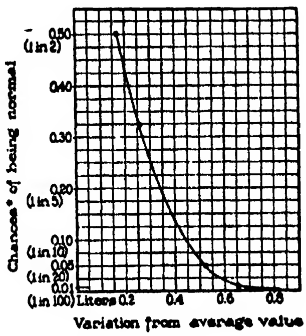

Fig. 5. A curve showing the probability that the observed cardiac index is normal expressed as variations from the average value in men over 40 years of age.

* The phrase used for this expression by Ezekiel (8) is the "probability of specified departure" from the average value.

Note: When in an individual the cardiac index differs 0.65 liter from the average the chances are only 1:100 that this measurement is normal.

per cent, while the consumption of oxygen fell 9.3 per cent. According to the straight line equation for the cardiac index, the decline was 7.3 per cent. The regression of index on age seems to be the result chiefly of the regression in consumption of oxygen.

The standard error of estimate for the men, 40 to 89 years, is 0.259 liter per square meter per minute. The chances of being average depend upon variation from average values based on the standard error of estimate (fig. 5).

Discussion. In view of the very small decline in the cardiac index from forty to eighty-nine years of age, it is clear that the heart can maintain

[4] If like changes occur in consumption and in utilization the cardiac index remains uninfluenced. Unequal changes in the two obviously affect the volume of the cardiac index.

under basal conditions, so far as this function is concerned, a fairly constant level of activity until very late in life. In one man at all events, of 101 years, it met a demand for the same amount of blood as is customary in younger men. Obviously greater experience of this function in men of this age group is necessary than has been gained so far.

It appears that the decline in index occurs not solely as a consequence of age but as a result of the decline with age of the consumption of oxygen. In calculating the "cardiac output" Grollman (1a) has assumed that the surface area provides a suitable object of reference. In any event experience has shown that the index is proportional to the surface area in the same sense as is the consumption of oxygen. These calculations have all assumed constancy in the area, structure, and function of the skin. With this reservation the results are taken to present values for the cardiac index in normal men according to their age. They indicate that the index declines 1.5 per cent per decade.

The changes which have been studied relate solely to the basal state. Naturally, it is not under such conditions that capability is measured. Capability is measured when the body operates under stress. What Meltzer called factors of safety are then brought into play. The influence of exercise, of abnormal affections and of disease requires to be studied before insight into the reserve capacity of the heart is secured. Obviously old men do not and perhaps cannot do what younger ones can—alterations in other circulatory functions may in a collateral way account for changes in the activity of the heart, even under basal conditions.

SUMMARY

1. The cardiac index has been measured in 100 normal men, 20 in each decade from 40 to 89 years, and in addition in two men of 91, and one of 101 years. The index declines 0.18 liter (7.3 per cent) during this period or in each decade 0.035 liter (1.5 per cent). The correlation is negative and is -0.1862 ± 0.0965. It is not significant. But if the entire series of 103 men is utilized it is -0.2039 ± 0.0944 and is significant. The rate of decline is almost the same.

2. The mean values in succeeding decades or half-decades suggest that the rate of decline of the curve is not straight but is arrested for a time after 55 years. To smooth the curve more observations are required. Variations in each age group exist as in younger men. In any 5 or 10 year group the maximum deviations from the respective means were 21.6 and 27.1 per cent. The coefficient of variation of the means varied from 8.3 to 16.6 per cent.

3. For the entire series (40 to 101 years) the mean is 2.26 liters per square meter per minute; the standard deviation is 0.26 (11.6 per cent of the mean) and the standard error of the mean is 0.026. For the evenly

distributed group of 100 men the results are unchanged; the mean value was 2.26 liters, the standard deviation 0.26 (11.5 per cent of the mean), the standard error of the mean 0.026, and the standard error of estimate 0.259 liter.

4. The decline in cardiac index follows chiefly upon the decline of the oxygen consumption since the influence of change in arterio-venous oxygen difference is relatively small.

5. Though the measurement of the index indicates slight decrease in the work of the heart under basal conditions, there remain to be considered other functions which are involved in changes in cardiac activity with age.

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THE ELECTROGRAM OF CARDIAC MUSCLE: AN ANALYSIS WHICH EXPLAINS THE REGRESSION OR T DEFLECTION

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I

Ever since the discovery that an electrical response is associated with the activity of certain tissues, physiologists have been interested in ascertaining the origin and nature of these currents. While these two subjects are closely associated, they are not identical. In the former belongs the effort to discover the physicochemical reactions in tissue that produce the electrical energy, in the latter, the endeavor to ascertain the character of the currents produced, their distribution and association with the other manifestations of activity. To the general physiologist the origin of the electrical response has been the subject of greater interest while the nature of the currents has been investigated only as a means to this end. But now that electrocardiography has come to play an important rôle in clinical medicine, a clear description of the electrical events which take place in a single cardiac muscle fiber as a result of activation is a matter of practical importance apart from any light it may throw upon the origin of these currents. Since this investigation sheds no direct light on the origin of action currents the history of that subject will not be discussed. Meanwhile it is desirable to consider briefly the state of knowledge of their nature—at least so far as heart muscle is concerned.

The experiments upon which this knowledge is based fall into two categories, those performed in moist air and those in which the tissue is in situ or is immersed in a vessel containing physiological salt solution which serves as an extensive conducting medium. However the

A preliminary report of this work was read before the American Society for Clinical Investigation in May, 1935.

experiments were performed, provided only that the muscle was uninjured, the response obtained consisted of two parts, a rapid primary deflection and a slower secondary or T deflection.

In the first group of experiments it was invariably found that when one electrode connected with a galvanometer was on active and the

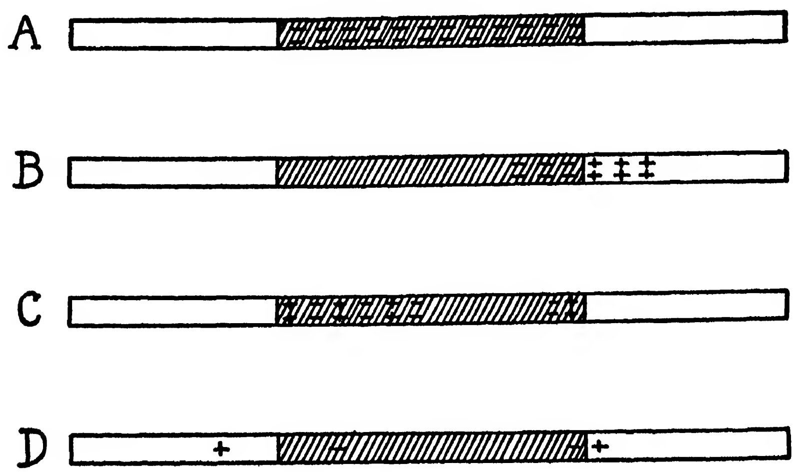

Fig. 1.—A diagrammatic representation is given of the various theories of the nature of the electrical manifestations of activity in cardiac muscle. In each case activity is spreading from left to right. The active region is shaded.

A. The negativity hypothesis. The entire mass of active muscle is represented as being negatively charged. The inactive muscle is neutral.

B. The theory of limited potential differences (Lewis). A small region where the muscle has just become active is negative and the immediately adjacent resting muscle is positive.

C. The doublet theory (Craib). As muscle becomes active it becomes the seat of doublets whose positive element is toward the resting muscle. When muscle regresses from the active state, it gives rise to doublets of opposite polarity.

D. Bipolar theory (Wilson, Macleod, Barker). Ahead of the advancing boundary between resting and active muscle is a positive pole and behind it a negative pole. Across the retreating boundary is a potential difference of reversed polarity but in this case the poles are farther apart.

other on resting muscle, the active muscle was negative with respect to the resting. Consequently, the first theory to gain general credence was the so-called negativity hypothesis or theory of distributed potential differences. According to this theory active muscle becomes negatively charged (Fig. 1A). While anyone familiar with electrical

phenomena from the physical point of view would have found many reasons for considering the theory untenable, it explained fairly satisfactorily the early experiments performed on simple muscle strips suspended in moist air.

It was not until Lewis[1] began his investigations of the electrocardiogram that this theory was seriously questioned. After making a critical study of the spread of the impulse over the heart with the organ in situ he discovered that he could not explain his observations on the assumption that all active muscle was negative relative to resting, for he found that the sign of the galvanometric deflection depended only upon the direction in which the impulse was at the moment spreading and not on the location of the mass of active muscle as a whole with respect to the mass of resting muscle. He put forward an hypothesis, therefore, which he called the theory of limited potential differences. The view was that only the muscle which had just become active was relatively negative and that only the inactive muscle immediately adjacent was relatively positive (Fig. 1B). This idea adequately explained all his experimental observations. Lewis did not push the idea beyond his immediate needs, however, and did not make use of it in his explanation of the secondary or T deflection. It is unwise to conclude what an author's view would have been about a situation which he did not discuss unless such a view follows unequivocally from definite statements in his published work. No effort is made, therefore, to carry Lewis's theory further than he himself carried it. For a time there seems to have been little interest in the subject. But in 1927, Craib,[2] working with cold-blooded and mammalian hearts immersed in large baths of physiological saline solution, made an accurate study of the electrical field surrounding active heart muscle, and demonstrated, though not for the first time, the necessity of applying the laws which govern the distribution of potential in volume conductors* to this problem. Because he found while

* By a volume conductor is meant an extensive tridimensional conducting medium. A jar of physiological saline solution, or the body of a patient or an animal is a conductor of this sort. Certain of the early investigators, notably Waller and Einthoven, understood that it was necessary to apply the laws which govern the distribution of potential in volume conductors when dealing with the heart in situ, and whatever simplifying assumptions they made they were careful

making these studies that he could assume the source of potential difference during systole to be a doublet† he proposed a doublet theory for the nature of the electrical manifestation of muscular activity. According to this theory, at the moment of activation, doublets develop in the tissue and endure there for a brief period. Later, as activity subsides, doublets of opposite sign appear and last a somewhat longer time (Fig. 1C). Results of experiments on muscle strips could be accurately predicted from this theory whereas predictions on the basis of the old negativity hypothesis could not be verified.

Finally, Wilson, Macleod, and Barker[3,4] made an extensive study of the laws which govern the distribution of potential in volume conductors and their application to the problems of electrophysiology. They treated the subject more generally than Craib had done and reduced their analyses to mathematical form. In this way they were able to plot curves that should be obtained if the conditions assumed prevailed and to compare these with electrograms obtained by experiment. There was very close agreement between their theoretical and their actual curve. Their conclusion was, "whatever may be the origin of the electric currents associated with the excitation wave, these currents are similar to those which would be produced if the crest of the excitation wave were immediately preceded by a source (positive pole) and followed by a sink (negative pole)." They suggested also that the secondary or T process is caused by a sink followed by a source, but that in this case the poles are farther apart (Fig. 1D).

The first (negativity) theory in the light of present knowledge is

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to justify. Later investigators for the most part, however, did not realize the necessity of the application of these laws. The report of Wilson, Wishart, and Herrmann[6] in 1926 was probably the first in more recent years to call attention to the fact that the body must be regarded as a volume conductor.

† A doublet is a positive and negative pole of equal strength located very close together, strictly speaking infinitely close together, i.e., a potential difference with magnitude and direction located at a point. The conception is a mathematical fiction devised to facilitate the application of mathematics to the solution of electrical problems. A potential difference which extends over a very small space behaves, of course, under certain circumstances, like a doublet; any potential difference can be represented by a combination of doublets. Craib may not have appreciated the fictitious character of doublets and the equivalence of a train of doublets to a potential difference extending over a space.

untenable. The difficulties with it have been pointed out by Craib[2] and by Wilson, Macleod, and Barker[3] and need not be dealt with at length, except to point out the nature of the fallacy involved. If a strip of muscle *AD* (Fig. 2) surrounded by moist air is stimulated at *A*, its active (shaded) portion is negative with respect to its resting portion. In other words, so long as the boundary between active and resting muscle (*X*) is between *B* and *C*, an electrode at *B* is negative with respect to one at *C*. The inference drawn was that this result signified that active muscle was negatively charged because inactive muscle was neutral (Fig. 1A). This, however, is only one of two possible explanations. An equally satisfactory and more probable one is that a potential difference exists at the boundary *X* between active and resting muscle (Fig. 1D as contrasted with Fig. 1A).

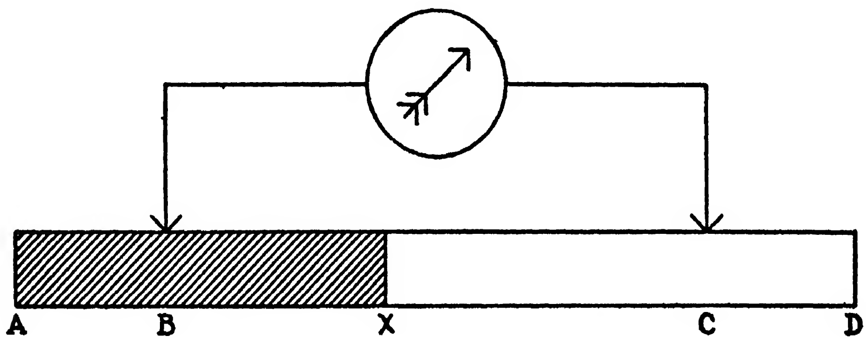

FIG. 2

Under the circumstances just described, with the muscle suspended in moist air, if a potential difference existed at *X*, the strip *BX* would act as an extension of the electrode *B* and, therefore, seem to be negative, and *XC* as an extension of *C*, and seem positive. In the case of a linear conductor, which a muscle strip suspended in air closely approximates, it is impossible to differentiate between the two explanations. If the muscle strip were immersed in a sufficiently large bath of saline solution, however, it could be ascertained which explanation is correct, that is, whether the entire mass of active muscle behaves as an extensive negative pole and the entire mass of resting muscle as an extensive positive pole, or whether there seems to be a localized positive and negative pole close together in the region of transition *X*. The propounders of the last three theories (Fig. 1

B, C, D) have done such experiments and have found the second situation to represent the facts. The three are not dissimilar. Each may in a sense be regarded as an extension and clarification of the preceding.

Lewis explained that his theory of limited potential difference was vague. Nevertheless he demonstrated that the excitation wave is preceded by a positive and followed by a negative region, and that both are of small extent. His failure to offer an explanation of the secondary or T deflection in terms of his theory was its greatest omission.

Craib's doublet theory explained consistently both QRS and T, but was purely qualitative and predicted little more than the sign of the deflections. In the case of the secondary (T) deflection his predictions were furthermore not well borne out by the experimental curves.

Wilson, Macleod, and Barker were more precise in their concepts, and their deductions were expressed in mathematical form so that they were able to predict the shape of the primary deflection with considerable nicety and to demonstrate the significance of the various inflections of the curve. This made it possible to ascertain from the recorded curve a fairly precise description of the electrical process which produced it. They did not attempt to predict the form of the T-wave in direct leads but were able to show by an indirect method that it bore a quantitative relationship to the QRS deflection and was produced by electrical forces of opposite sign.[4,5] In a different way each theory has demonstrated that as the excitation wave spreads, it is accompanied by a positive and a negative region each of limited dimensions. The way in which each has pictured the behavior of the electric forces is different, but the differences are superficial, and all are in agreement as to the fundamental nature of the process. Both Craib, and Wilson, Macleod, and Barker have indicated that the secondary or T deflection is probably produced by forces of opposite sign to those that cause the QRS, and that it has to do with the recovery from the active states. But the nature of this process is still far from clear. The object of this research is to describe intimately the concepts of activity in general and the electric phenomena which accompany it, to relate the one to the

other and thereby to explain more satisfactorily the nature of the secondary (T) deflection.*

II

This investigation deals primarily with an explanation of the secondary or T deflection. But since it makes use of a new method of analysis, it seems best for the sake of clarity, to deal briefly with the electrical process as a whole.

The ideal experiment for a study of this kind is one in which the physical circumstances are sufficiently well understood and sufficiently simple so that potential changes occurring in a given segment of active tissue can be easily and unequivocally inferred from the record obtained. As has been mentioned, certain information can be gained from experiments in which appropriate tissue is immersed in an extensive conductor (a saline bath) that is not forthcoming from experiments performed with preparations suspended in air. There is a distinct disadvantage, furthermore, in placing both electrodes on the tissue being studied, since it is then impossible to tell whether a given deflection is produced by a positive effect at one electrode or a negative effect at the other. As Wilson, Macleod, and Barker[3] showed, this difficulty can be avoided by placing one electrode, the exploring electrode, on the tissue under observation, and the other, the indifferent electrode, in the conducting medium at a sufficient distance from the first so that potential differences arising in the active tissue will produce undetectable, because so small, changes in potential in it. The record then describes the potential changes occurring at

* The work of Eyster, Maresh, and Krasno (Am. J. Physiol. **110:** 422, 1934) and Krasno, Eyster, and Maaske (Ibid. **114:** 119, 1935) has not been discussed in this paper because their work deals with the potential changes in the heart as a whole as judged by indirect leads rather than the electrical events occurring in a single fiber. These authors have made use of a doublet concept but the doublets which they assume are the resultants of all the potential differences existing anywhere in the heart at a given moment. Theirs clearly is a different concept from the one used here where doublets are used to explain the potential differences existing in single muscle fibers. For these reasons the work of the authors mentioned requires no detailed analysis and comparison with the discussion in this paper. This excellent work will be reviewed in a more appropriate connection.

the point in the active tissue upon which the exploring electrode has been placed. It is, in addition, necessary to know the configuration of the muscle and the way in which the active process passes over it. While a long narrow strip of tissue in which impulses spread from one end to the other would be ideal, it is not practicable to cut such a strip from the heart since it is especially necessary to avoid the complication of electrical effects attributable to injury.

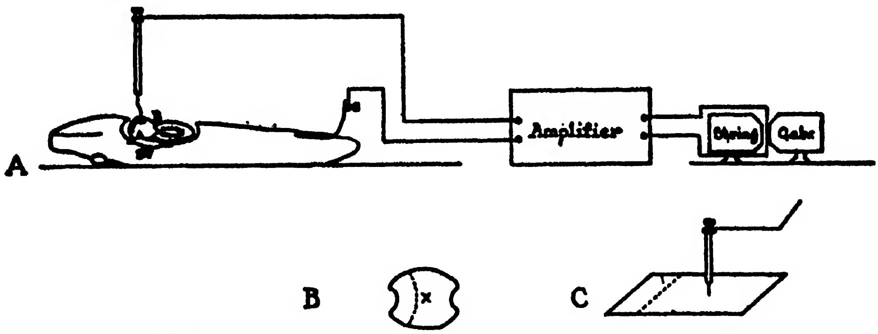

FIG. 3.—A. A schematic arrangement is shown of the method of obtaining electrograms from the uninjured auricle of the frog.

SV = sinus venosus.

A = junction of anterior wall of auricle with sinus venosus.

B = junction of anterior wall of auricle with ventricle.

B. This illustrates the approximate shape of the anterior wall of the auricle. The dotted line represents the position of junction between active and resting muscle at a given moment.

X is the position of electrode.

C. A uniform sheet of muscle is shown in which activity is spreading from left to right. The dotted line indicates the position of the boundary between resting and active muscle at a given moment. This figure illustrates what Wilson, Macleod, and Barker refer to as "parallel excitation."

In the auricle of the Louisiana bullfrog, *Rana catesbiana*, the impulse spreads in such a way that the conditions on its anterior surface approximate those in a simple strip of muscle. The impulse arises in the sinus venosus (Fig. 3A, *SV*) and spreads in the auricle from *A* toward *B*. If this anterior portion of the auricle were flattened out its shape would resemble Fig. 3B. At a given moment the division between active and resting muscle might occupy the position of the dotted line. The point *X* indicates the position of the exploring electrode. This arrangement approximates the situation which

Wilson, Macleod, and Barker designate as "parallel excitation"[3] (Fig. 3C). They have shown that the electrogram under these circumstances differs only quantitatively from one produced in a narrow strip.

The impulse arising in *SV* spreads to the ventricle also over the posterior auricular wall on the dorsal aspect of the heart. The potentials produced in this bit of muscle are small and sufficiently distant from the exploring electrode so that their effect is negligible.

In performing an experiment the animal was first pithed to prevent muscular twitching. The chest and pericardium were then opened and in some experiments heart-block was produced by pulling tight a ligature laid around the A-V groove. Since it was necessary to approximate the conditions of an extensive uniform conductor, good contact with all the surrounding tissues was maintained by filling the body cavity with saline solution when necessary. At the site where the exploring electrode was to be placed a small patch of the epicardium was dissected off without injuring the underlying muscle. A small exudation of fibrin then caused the electrode, which consisted of a piece of thread protruding from the end of a silver tube, to adhere to the muscle without slipping during contraction. The indifferent electrode, a strip of silver, was placed beneath the skin of the hind leg.

Since the resistance of the exploring electrode was of necessity high, a single stage direct current amplifier was used in conjunction with the string galvanometer. The maximum gain of this amplifier was approximately 10. It had, however, to be stable and more than usually "quiet" because of the extreme sensitivity of the recording instrument. The sensitivities actually employed were deflections of 1 to 2 cm. per millivolt. Figure 3A is a diagram of the arrangement in a typical experiment.

When the exploring electrode was placed midway between *A* and *B* (Fig. 3A) the curve obtained was like the recorded curve of Fig. 10C. It may be considered to consist of two parts, a primary deflection which is rapid and diphasic (positive-negative) and a secondary deflection which is slower and monophasic (positive). An approximately isoelectric period separates these two deflections. The rapid primary deflection of this electrogram corresponds to the QRS group of the electrocardiogram and the secondary deflection to the T-wave.

When the exploring electrode is placed near the auriculoventricular junction the curve obtained is seen in Fig. 11C. This curve differs from the preceding in that the positive phase of the primary deflection is much larger and the negative phase smaller. The portion of the curve separating the primary and secondary deflections is above the isoelectric line, and the secondary deflection is smaller. Finally, if the exploring electrode is placed nearer the sino-auricular junction, the electrogram obtained (Fig. 12C) differs from the one obtained from the central region in an opposite way from the one just described. The positive phase of the primary deflection is smaller and the negative phase larger, the portion of the curve which separates primary and secondary deflections is below the isoelectric line and the secondary deflection is larger.

To explain the form of these curves it is first necessary to return to a consideration of certain theoretical matters.

III

Before considering the electrical events which take place in a muscle fiber during activity it will make for clarity if the general process of activation is described.

If a long muscle fiber (Fig. 4A) is stimulated at the left end, the excitation process spreads toward the right at a certain velocity which may be designated V_e. When it reaches each minute muscle element such as x_1, x_2 this element becomes active and remains so for a time (T) and then returns to the resting state. During the time (T) that x_1, x_2 is in some stage of activity, the excitation process will have progressed along the fiber. The distance traveled will be TV_e. At any instant after stimulation a length of muscle (L) equal to TV_e is, therefore, in some stage of activity. That is what is meant by the length of the active process. The total time during which the segment is active (T) may be divided into three parts, the period during which its activity is increasing (T_1), the period during which it is fully active (T_2), and the period during which its activity is regressing (T_3). During these intervals T_1, T_2, T_3 the excitation process will have traveled the distances $T_1 V_e$ (*AB*, Fig. 4A), $T_2 V_e$ (*BC*, Fig. 4A), and $T_3 V_e$ (*CD*, Fig. 4A). These are, respectively, the lengths of the stages of increasing, full, and decreasing activity. Conse-

quently, that portion of a muscle which at any moment is active may be represented as consisting of three parts, a region where activity is increasing (*AB*, Fig. 4A), a region whose activity is fully developed (*BC*, Fig. 4B), and a region where activity is regressing (*CD*, Fig. 4B). During activation a tripartite process of this kind may be pictured as passing along the muscle at the velocity V_e. In the discussion which follows, therefore, the excitation process will be represented as in Fig. 4B. A knowledge of the actual values of

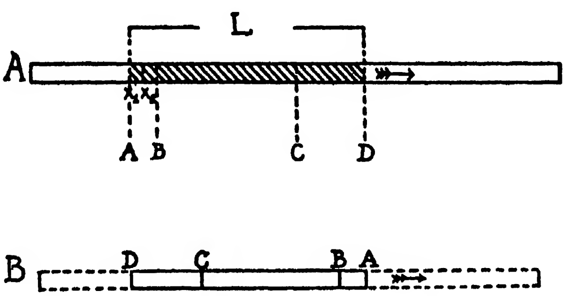

FIG. 4.—A. A muscle strip is stimulated at the left end. The shaded area represents the extent to which the excitation process spread during the time that the muscle in x_1x_2 was in some state of activity.

AB indicates the distance traveled by the front of the active process while activity was increasing in x_1x_2.

BC indicates the distance traveled by the front of the active process while activity was full in x_1x_2.

CD indicates the distance traveled by the front of the active process while activity was regressing in x_1x_2.

B. The tripartite process of activation which is traveling along a muscle strip. The muscle from *A* to *B* is increasing in activity, from *B* to *C* is fully active; and from *C* to *D* is decreasing in activity.

T_1, T_2 and T_3 and V_e constitutes a fairly accurate description of the excitation process. How these are ascertained from a recorded curve will be related in a subsequent report. In this discussion, arbitrary and appropriate values have been assigned to them.

V_e as has been said is the velocity of excitation. This (velocity of excitation) is equal to the velocity of the progress of complete recovery, V_r, if T is the same for every muscle segment. In other words, the velocity of the right end of the shaded area (Fig. 4A) is equal to the velocity of the left end If T were to become progressively

shorter, however, from left to right, V_r would be greater than V_e, for the length of the active process would be shortening as it progresses; and if T were to become progressively longer from left to right, V_r would be less than V_e, for the process would be lengthening as it progresses. If a uniform fiber is in a uniform environment, however, V_e will be uniform, T will be the same for each element, and V_e will equal V_r. It is these simple circumstances which are assumed to be present in the analyses which follow.

The electrical events accompanying activity may now be described. There is good experimental support for the belief[2,3] that a potential difference exists at the junction between active and resting muscle, and that this potential difference is such that the positive pole is toward the resting muscle. Since active muscle must in some way

FIG. 5.—A diagram representing a muscle strip is shown in which the active process is progressing toward the right. The shaded area is the active portion at a given moment. The depth of the shading roughly indicates the degree of activity.

A indicates junction of resting and active muscle.

BC indicates region of decreasing activity.

differ from resting muscle, and since at the junction between any two substances that differ from each other chemically or physically, a potential difference may exist, it is probable that in the present case the potential difference occurs because of the difference in composition between active and resting muscle. In the sense that the transition from resting to active muscle constitutes a change in phase, the action current may be considered as resulting from a phase boundary potential. What the ionic mechanism may be, whereby this potential difference is produced, is for the present irrelevant.

A long muscle fiber has been stimulated at the left end and that portion of it which is at the moment active is shaded (Fig. 5). The depth of the shading roughly represents the degree of activity. For convenience the active portion has been divided into small equal segments. The transition from resting to active muscle at *A* is

represented as being abrupt, but that in the reverse direction, in the region BC, as taking place in four steps. If the assumption which has been made is correct, a potential difference exists across the boundary A, such that its forward looking aspect is positive and its backward looking aspect negative. Across any boundary between A and B no potential difference exists, however, for the constitution of the muscle on one side is precisely the same as on the other, both fully active. In the case of the boundary at B, however, the muscle to the left is slightly less active, than that to the right. Consequently, a potential difference should occur here of smaller magnitude than that at A, and of opposite direction. A similar situation exists at each of the segment boundaries in this region, including the one at C. The sum of all these potential differences must, of course, be equal in magnitude to the single one at A, for one transition is simply the reverse of the other. A transition from resting to active muscle, or vice versa, in reality does not occur abruptly or even in a series of distinct stages, but gradually. In other words, if the number of segments in the region BC were greatly increased, the facts would be more correctly represented. In this case each individual potential difference would be less, for the sum must remain the same. In similar fashion, instead of an abrupt transition at A, a gradual one of short duration (in many short steps) would represent the course of events more accurately.

Since the number of segments in the transitional regions may be increased without limit, thus approximating the gradual transition with any desired degree of accuracy, and since a doublet is defined as a positive and a negative charge infinitely close together, muscle in the transitional state may be considered to be the seat of a train of doublets (Fig. 6). It is well known that such a train is equivalent, furthermore, to a single positive and a single negative pole located at either end of the train (Fig. 6). This leads to a generalization of importance, namely, that any bit of muscle, such as x_1, x_2 (Fig. 6) in the transitional state may be considered to have a positive pole located at its less active and a negative pole at its more active end.

As has been pointed out (Fig. 4B) the active process may be graphically represented as a rectangle divided into three parts corresponding to the phases of increasing activity, full activity, and decreasing

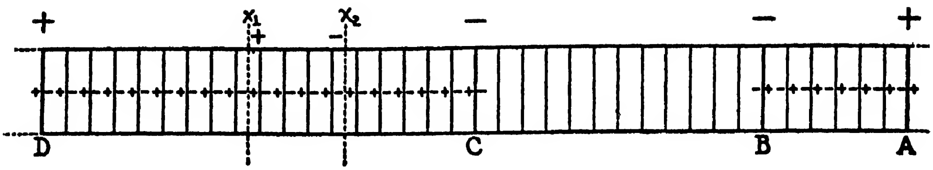

FIG. 6.—A muscle strip is shown in which the active process is progressing toward the right. The active region is divided into small segments. Each is supposed to be in a uniform state of activity throughout its extent. In the region *AB* activity is increasing and each successive segment is more active than its neighbor to the right. In the region *CD* each segment is less active than its neighbor to the right. A potential difference exists between every two adjacent segments if their states of activity are different. In the transitional regions trains of doublets thus arise. These trains are equivalent to a positive and negative pole separated by their length. The total change in potential is the same for both transitions. Thus a given length of muscle x_1x_2 contains a smaller proportion of the total if it is in the region *CD* as depicted than if it were in the region *AB*.

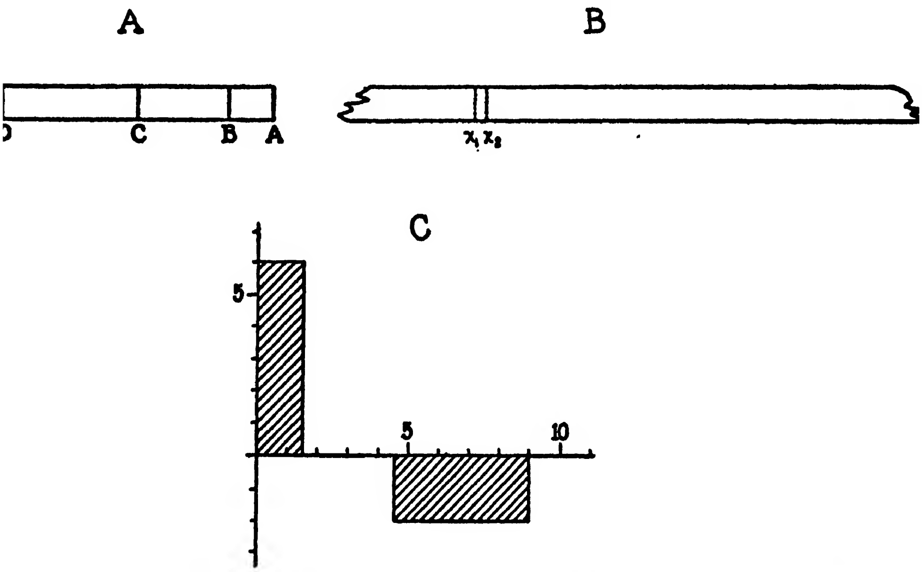

FIG. 7.—A diagram is shown indicating how the process of activation passes over a muscle strip.

A is the process of activation schematically represented.

AB is the stage of increasing activity.

BC is the stage of full activity.

CD is the stage of decreasing activity.

B. Muscle fiber over which the active process *A* is to pass. x_1x_2 is small segment of muscle whose electrical activity is plotted in *C*.

C is a graph illustrating the electrical phenomena attendant upon the activation and recovery of x_1x_2. The abscissas are arbitrary units of time and the ordinates appropriate units of potential.

activity (Figs. 6 and 7A). Since the same change in state, and therefore the same change in potential, is accomplished in the region AB as in the region CD, it must follow that a given short length of muscle, such as x_1, x_2 (Figs. 6 and 7B), will contain a greater proportion of the total potential difference existing between resting and active muscle when it is in a region where activity is increasing than when in a region where activity is decreasing. The magnitude of the potential difference existing in x_1, x_2 during increasing activity is, furthermore, as much greater than that during decreasing activity as AB is shorter than CD.

On the basis of this analysis it is possible to illustrate graphically the electrical events taking place in a small segment of muscle such as x_1, x_2 during activation and recovery (Fig. 7B). While activity is increasing in the region x_1, x_2 the muscle near x_1 is more active than that near x_2 and the segment is consequently the source of a potential difference whose negative pole is at x_1 and whose positive pole is at x_2. This magnitude is plotted above the axis of abscissas and is given the arbitrary value of 6 (Fig. 7C). Its duration is equal to the duration of the phase of increasing activity. In other words, the electrical activity of x_1, x_2 during the phase of increasing activity is represented by a rectangle whose height is 6 and whose width is equal to AB. While the muscle throughout x_1, x_2 is fully active, no potential difference exists within these confines, so for a period equal in length to BC there is no quantity to be represented. But when the muscle in x_1, x_2 begins to decrease in activity, a potential difference again develops and persists for a period equal in length to CD. During this period the muscle in the vicinity of x_1 is less active than that in the vicinity of x_2 so that a positive pole will exist at x_1 and a negative one at x_2. Since the potential difference is opposite to that present during increasing activity, it is plotted below the axis of abscissas. A rectangle results, therefore, one-third as tall and three times as long as the one representing the electrical activity during the phase of increasing activity for CD is three times as long as AB. Before proceeding to the consideration of an actual experiment, another matter must be considered.

An exploring electrode is now represented in relation to the long muscle fiber (Fig. 8). For reasons previously pointed out, it is unnecessary to consider the potential changes of the indifferent electrode

(the one at a distance from the active tissue). Since the muscle is immersed in an extensive conduction medium of uniform conductivity, the law[3] $\left(E = \frac{\mu \operatorname{Cos} \theta}{R^2}\right)$ applies, where E is the effect upon the electrode produced by any given dipole, R the distance of the electrode from the center of the dipole and θ the angle between the line from the tip of the electrode to the center of the dipole and the positive end of the axis of the dipole.

This equation expresses the obvious fact that any potential difference anywhere in the medium has an effect upon the electrode, that the more distant it is the less its effect and, furthermore, that its orientation with respect to the tip of the electrode also has an effect. When the positive element is closer (Fig. 8A) the effect is positive;

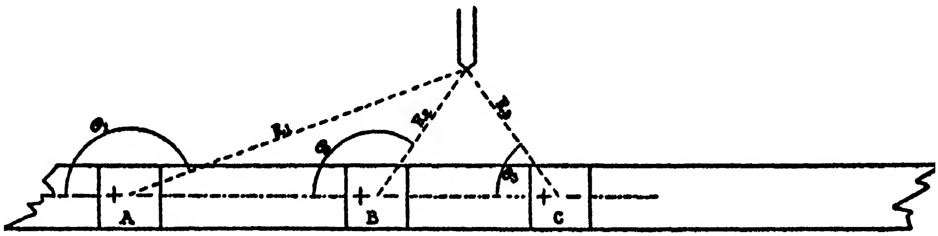

FIG. 8.—The effect is shown of variously placed doublets upon the electrode. *A* has less effect than *B* but both effects are negative. The effect of *C* is equal in magnitude to that of *B*, but positive.

when the negative is closer, negative. By means of this formula the magnitude of the effect upon the electrode of any potential difference existing in any portion of the muscle can be calculated.

In the center of a long muscular fiber, divided into squares, is a cross (Fig. 9) which serves to indicate the position of the exploring electrode, the tip of which is supposed to be one unit of length above the plane of the paper. *ABCD* represents the familiar arrangement of the process of activation and recovery. The effect upon the electrode of the electrical events which occur in the first square while its activity is increasing can be ascertained by applying the formula $E = \frac{\mu \operatorname{Cos} \theta}{R^2}$.* This effect on the electrode is represented in the

* In making the actual calculations for this graph and the ones to follow, the width of one square was used as the unit of length and μ given an arbitrary but

figure by the first shaded rectangle below the muscle fiber (the first from the left). Its height corresponds to the electrical effect produced upon the electrode and its length to the duration of this effect. The duration will, of course, be equal to AB, since it is assumed that the velocity with which the process travels is one unit of distance in one unit of time. Once this element has become fully active it will no longer exhibit any potential difference. Thus, for the next seven units of time, no potential difference will exist here. But during the four and one-half units of time while activity is regressing (equal to CD) it again becomes the source of a potential difference, but this time of opposite polarity. This effect is represented by the first long narrow shaded rectangle above the muscle fiber. Since the regression process is three times as long as the period during which activity is increasing, the potential difference produced in a given segment in the first case is one-third that produced in the second. Consequently, this rectangle is one-third as high and three times as long as the one plotted for the period of increasing activity. Furthermore, since the positive pole is always toward the resting muscle, and the negative toward the active muscle in the case of increasing activity, the positive pole will be nearer the electrode and the effect upon it, therefore, positive, and in the case of decreasing activity, the negative pole will be nearer the electrode and the effect upon it negative. Since in electrocardiography the galvanometer is always so arranged that a negative effect produces an upward deflection, the negative effects are plotted above the base line (muscle fiber) and positive effects below.

Next, a similar plot is made of the electrical effects produced in the second square. The rectangles in this case are entirely similar to those for the first segment but both are of greater height, since the segment is closer to the electrode.

In the figure, rectangles have been constructed for each of ten segments, five to the left of the electrode (hatched), and five to the right (hollow). The effects of the segments to the right of the electrode must, obviously be of opposite polarity to those to the left, so that in this case the effects of increasing activity will be plotted

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appropriate value so that the resulting graph would be of a proper size. Negative effects are plotted above the line and positive effects below, to correspond to the conventional method of recording electrograms.

above the line, and those of decreasing activity below. All the electrical effects produced are represented in their proper time relations. The first, that of increasing activity, starts when the excitation

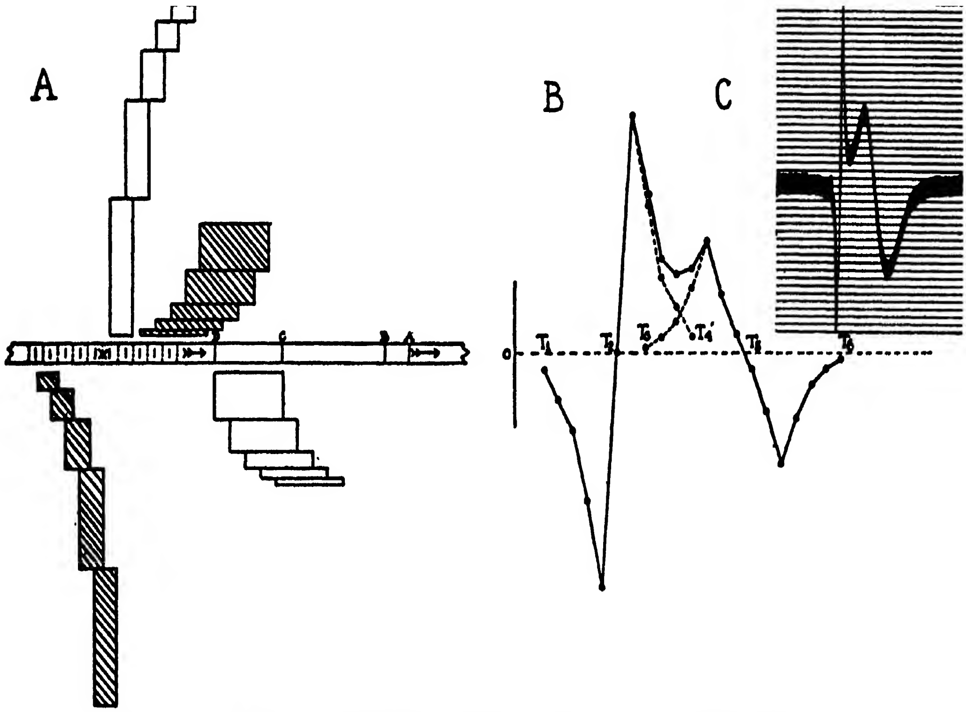

FIG. 9.—A is a plot of the electrical effects of each of ten muscle segments, five on either side of an electrode which is located one unit of distance toward the reader from *X*. *ABCD* is the excitation process; *AB* the stage of increasing activity, *BC* the stage of full activity, and *CD* the stage of decreasing activity.

B is a graph of the resultant electrical effects for each moment of time during the passage of the excitation process over the ten muscle segments represented in A—i.e., a synthetic electrogram. The dotted line starting at T_3 is a graph of the electrical effects of the regression of activity in the muscle elements to the left of the electrode plotted alone, and the dotted line ending at T_4 represents the electrical effects of accession of activity in the muscle elements to the right of the electrode plotted alone. (The full line in this region is a combination of these effects.)

C is an actual record taken from the central region of a frog's auricle after the preparation had been warmed.

process reaches the muscle element and endures so long as this transitional (increasing) state lasts. The second, that of decreasing activity, is plotted not over the muscle element in which it originates,

but at the position corresponding to the *time* at which it occurs. Consequently, it is only necessary to take the sum of all the effects present at a given instant to obtain the height of the electrogram at this instant. Thus, any line drawn perpendicularly to the base line (muscle fiber) will cut one or more rectangles. If the segments of the intercepted rectangles are measured and added algebraically, the ordinate of the electrogram for that instant is obtained. Figure 9B is the theoretical electrogram so plotted. It will be seen that the *O* value of the ordinate at T_2 is the result of the sum of two equal but oppositely directed potential differences and not the nonexistence of a potential difference at this time. In the region to the right of T_2, effects of the regression of activity in segments to the left of the electrode are combined with effects of increasing activity in elements to its right, with the result that the curve does not return to the base line in this region. The dotted lines in the figure represent the effects of the accession and regression of activity in this region of overlapping, plotted independently. It is obvious from the figure that the electrogram is the sum of two diphasic curves, the first the result of the onset of activity, and the second the result of its regression, separated by an interval equal to the period during which each segment of muscle is fully active. It is to the second of these curves that particular attention will be directed, for adequate analyses of the former have already been made.

The recorded curve (Fig. 9C) was obtained from the frog's auricle by the method already described. Its similarity to the theoretical one is obvious. All the essential features of the recorded curve are present in the theoretical one. This particular curve was obtained from the central portion of the auricle after the preparation had been heated by flushing it with warm saline solution. It was chosen for the first analysis because it exemplifies the process in a more general way than do the curves taken under more nearly normal conditions which will be discussed presently. Craib, and Wilson, Macleod, and Barker believed furthermore, from their studies of the primary deflection, that the secondary or T deflection should be of this shape. But if such curves had been obtainable under ordinary circumstances, correct analyses of the secondary deflection undoubtedly would have been made long ago.

In the earlier part of this study the first rapid deflection (QRS) of the electrogram or electrocardiogram has been referred to as the primary, and the slower and later (T), as the secondary deflection. These terms are not satisfactory and were merely used as a convenient means of referring to the parts of the curve. But now that a probable causal relationship has been shown to exist between the first rapid diphasic curve (Fig. 9, $T_1T_2T_4$) and the accession of activity and the second slower diphasic curve (Fig. 9, $T_3T_5T_6$) and the regression of activity, they will hereafter be designated as the accession deflection and the regression deflection.

It has been assumed that a muscle fiber is long enough to include all stages of activity at one time. But the conditions so far discussed do not represent the normal state of affairs. Obviously, since each small segment of muscle goes through every phase of activity, it is not at all necessary that fibers be long enough to contain at the same time, segments in each and every state from rest to full activity, and back again. It is permissible, therefore, to consider what may happen in a piece of muscle much shorter than the process of activity. In Fig. 10A is represented a short muscle fiber ten units long, the electrode located at the center but removed one segment's length from the plane of the page toward the reader. To the right, the excitation process is schematically represented. In the region *AB*, activity is increasing, in *CB*, it is fully developed, and in *CD*, it is decreasing. *CD*, the regression process, is more than twice as long as the muscle fiber. It is, of course, understood that length of process means merely the *time* during which activity is decreasing in a single muscle element, times the *velocity* with which the process travels.

If the electrical effects of each element in this short muscle are plotted as in the case of the long muscle fiber previously discussed, a graph similar to that in Fig. 9 is obtained (Fig. 10A). Figure 10B is the theoretical electrogram derived from the graph. The recorded curve (Fig. 10C) with which it is to be compared was obtained from the central region of the frog's auricle at room temperature, that is to say, under ordinary experimental conditions.

It will be noted that the electrogram, as before, is the sum of two diphasic curves, one produced by the electrical effects of increasing activity, and the other by the electrical effects of decreasing activity.

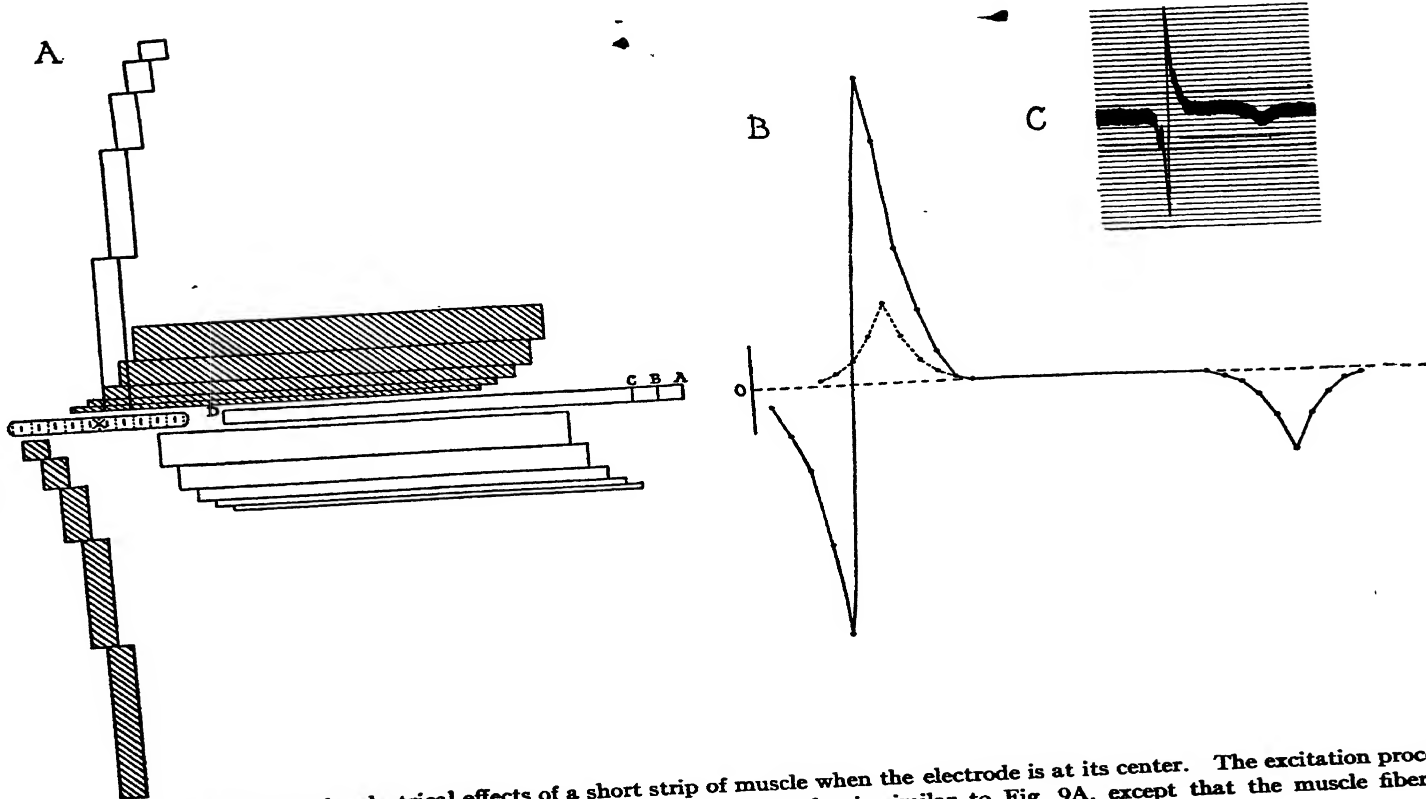

Fig. 10.—A is a plot of the electrical effects of a short strip of muscle when the electrode is at its center. The excitation process is represented by *ABCD*. It is much longer than the fiber. This plot is similar to Fig. 9A, except that the muscle fiber is short and the relationships between the phases of activity are different.

B is the synthetic electrogram obtained from A. The dotted line is the effect of the regression of activity in the muscle elements to the left of the electrode plotted alone in the region where they are combined with the effects of increasing activity.

C is an actual curve obtained from the central portion of a frog's auricle under ordinary experimental conditions.

Where the effects of decreasing activity (hollow rectangles) overlap those of increasing activity (shaded rectangles), the dotted line represents the effects of decreasing activity plotted alone. In this case it has been assumed that the muscle remains fully active for a short time only, so that the regression process (the effect of decreasing activity) begins very shortly after the accession process. The first phase of the regression process is, in these circumstances, entirely concealed in the larger accession process. This combination of effects necessarily distorts the accession deflection somewhat, rendering it asymmetrical. Since the portion of the regression process which is combined with the accession is an upward deflection, it slightly reduces the depth of the first downward phase of the accession deflection and augments the height of its upward phase. It is noteworthy also that while there is a long isoelectric region separating the first upward and second downward deflections of the regression process, this is the result of a balance between equal and opposite effects in muscle units to either side of the electrode, and does not indicate that no electrical effects are present during this period. This point will be made clearer in the next two experiments.

If, instead of placing the electrode over the center of the muscle strip, it is placed nearer to one end (the end at which the impulse arrives latest) as in Fig. 11A, a distinct change in the type of curve obtained takes place. The graph of the electrical effects produced and the theoretical electrogram are constructed just as in the previous cases. The actual curve with which the theoretical electrogram is to be compared was obtained from the anterior surface of the frog's auricle near the auriculoventricular junction. The similarity between the theoretical and actual curves is again marked. The curve does not return to the base line after the end of the second phase of the accession process, but remains above the isoelectric line for a time and finally dips slightly below it. In this case, the sums of the electrical effects of the regression process in the muscle elements on either side of the electrode are unequal so that the curve does not coincide with the isoelectric line between its upward and downward peaks.

The reverse effect is obtained by placing the electrode near the other end of the muscle strip, that is to say, near the end at which

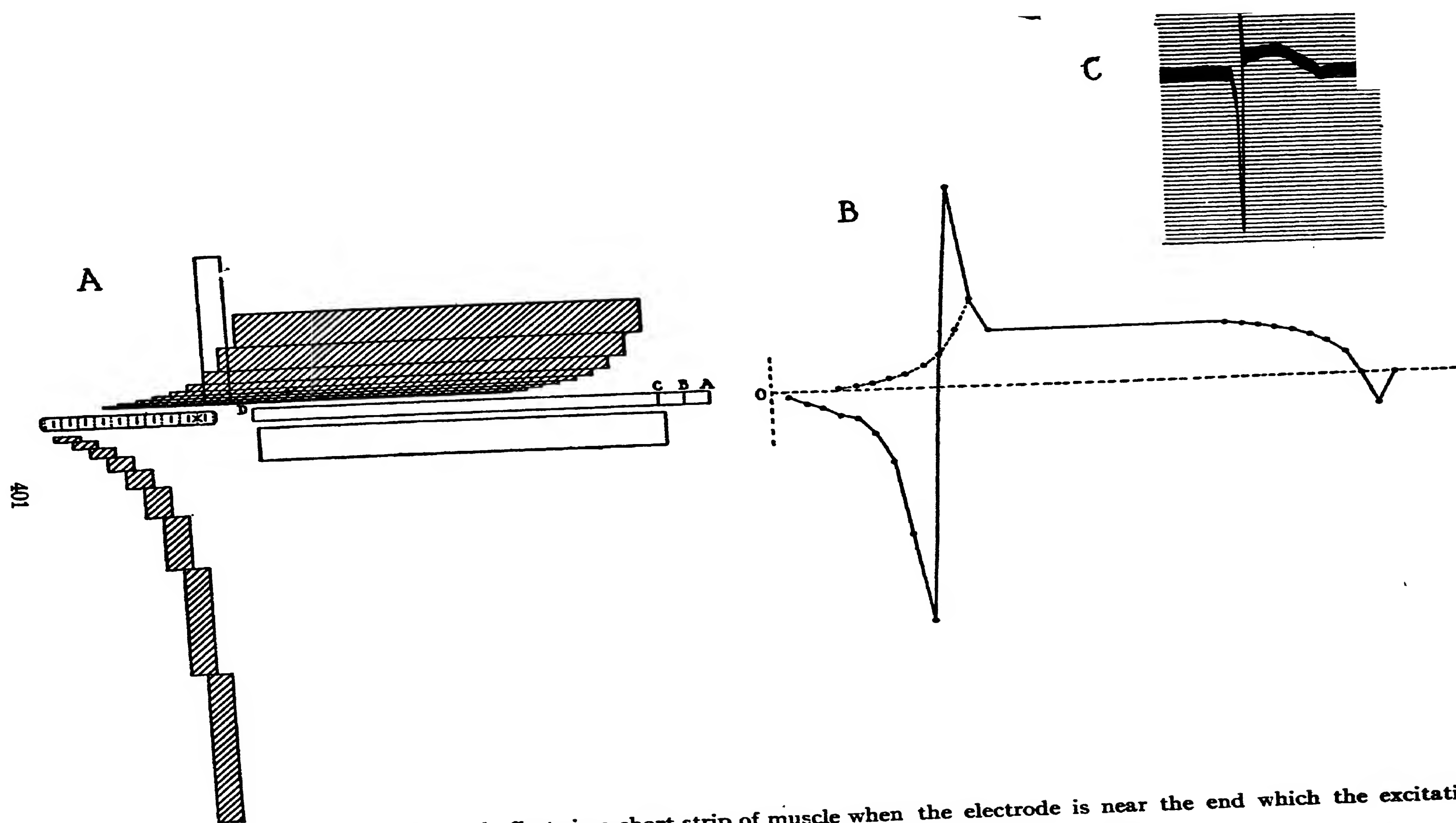

FIG. 11.—A is a plot of the electrical effects in a short strip of muscle when the electrode is near the end which the excitation process reaches last. *ABCD* is the excitation process. It is the same as in 10A.

B is the synthetic electrogram derived from A. The dotted line represents the effect of the regression of activity plotted alone.

C is an actual electrogram from a point on a frog's auricle near the auriculo-ventricular junction.

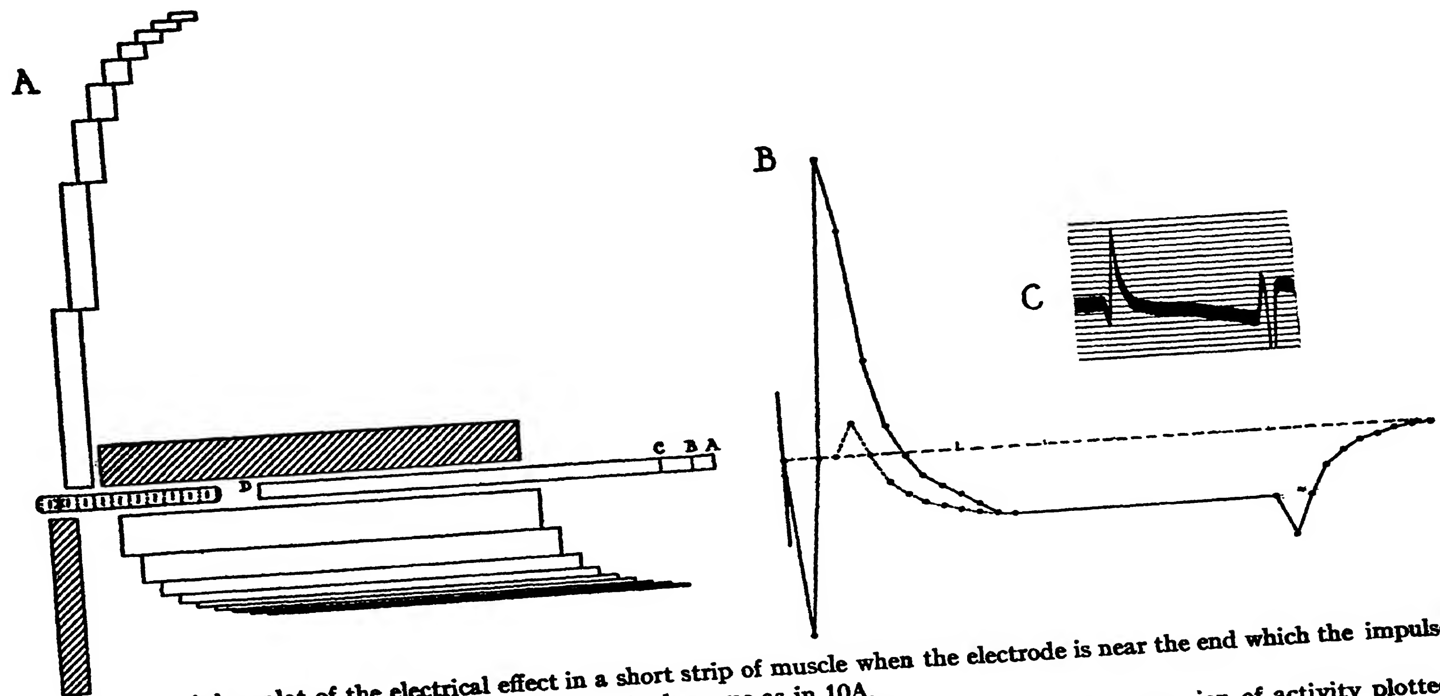

FIG. 12.—A is a plot of the electrical effect in a short strip of muscle when the electrode is near the end which the impulse reaches first. *ABCD* is the excitation process. It is the same as in 10A.

B is the synthetic electrogram derived from A. The dotted line represents the effect of the regression of activity plotted alone.

C is an actual electrogram from a point on a frog's auricle near the sino-auricular junction.

the impulse arrives first (Fig. 12A). The recorded curve which is to be compared with the theoretical electrogram was taken with the exploring electrode near the sino-auricular junction. This record is less satisfactory than the curves illustrating the other cases because its last part is distorted by the occurrence of a ventricular beat, and because in order to expose the sino-auricular junction, the heart was pulled out of its normal position. But the expected features of the curve can be identified and are quite similar to the theoretical electrogram. In this case the portion of the regression process between the two peaks is below the isoelectric line because the equilibrium between the forces on the two sides of the electrode, acting during this interval, has been unbalanced in the direction opposite to that in the previous case.

DISCUSSION

It is apparent from these analyses that the regression deflection is, as Craib and Wilson, Macleod, and Barker believed, a curve of opposite phase to that of the accession deflection. Its first phase may be concealed within the accession deflection and between its upward and downward peaks there may be a portion parallel to the base line. Craib's qualitative reasoning was too indefinite and Wilson, Macleod, and Barker's mathematical analysis too cumbersome to bring out these facts clearly. Discussion of the quantitative relationships between accession and regression processes will be left for a future time but it is obvious that such relationships exist and that an analysis of the T deflection of the electrocardiogram by means of the areas included by the complexes, such as Wilson, Macleod, and Barker[6] made, is appropriate.

Since there is evidence to indicate that the form of the electrogram is essentially the same for all forms of cardiac muscle, and since the time relationship between accession and regression processes is similar in electrograms (direct leads) and electrocardiograms (indirect leads), it is probable that part of the regression deflection of the electrocardiogram is really concealed in the accession deflection (QRS) and what has been called the S–T interval, is actually part of the regression deflection. That the S–T segment is an approximately straight line and either coincides with the isoelectric line or is parallel to it depends

on the fact that the length of the regression process is longer than the course over which it travels. Coincidence of this portion of the curve (S–T) with the isoelectric line is, of course, fortuitous. If a large number of electrocardiograms is inspected, it will be found, in fact, that the coincidence is seldom exact. According to this view, what has usually been called the T-wave is only the last phase of the regression deflection.

In most discussions of this kind some mention is made of the membrane theory of Bernstein. The theoretical concepts in the early part of this paper are quite different in purpose from those of Bernstein. They do not, as do his, attempt to explain the entire mechanism of stimulation and conduction, but aim only to elucidate one phenomenon, the action current. Whereas Bernstein created an ingenious but imaginary construction which might account for the phenomena observed, the argument here presented consists of logical deductions from facts experimentally ascertained, according to accepted physical methods. Since most of these ideas are quite different therefore from any held by Bernstein when he described his theory, it seems inappropriate to attempt to correlate them with it.*

The assumption has been made that active and resting muscle differ in their constitution and that the contact potential between them gives rise to the action current. The first statement will hardly be denied and the second, since it is an undisputed fact that a potential difference exists between active and resting muscle, becomes self evident if the term contact potential is used in its broadest sense, and no attempt is made to define the possible mechanism by which it is produced. Consequently the argument which has been developed is independent of whatever may be discovered about the significance of minute anatomical structures and the chemistry of activation and recovery. It can shed no direct light on these subjects but may be able to furnish criteria useful in their investigation for it follows from the causal relationship shown to exist between the stages of increasing and decreasing activity and the accession and regression deflections of the electrogram that it is possible to follow the course of the re-

* It is true that the potential difference between active and resting muscle can be accounted for by the membrane theory when properly interpreted[8] but the method used in the forepart of this paper is more direct and less confusing.

actions, as yet unknown, which constitute the processes of activation and recovery.

SUMMARY

1. A method for recording electrograms from the uninjured frog's auricle has been described which approximate those obtainable from a simple strip of muscle.

2. Starting with the observation that a potential difference exists between active and resting muscle, an analysis of the processes of activation and recovery has been made. Based upon this analysis, a graphical method for the construction of a complete theoretical electrogram has been devised. Any assumptions regarding the properties of the excitation process may be made and the appropriate theoretical electrogram plotted. When a theoretical and an actual electrogram accurately correspond, the properties of the excitation process in the muscle which produced the actual curve are presumed to be similar to those assumed in constructing the theoretical one.

3. The regression, like the accession deflection, is expressed in a diphasic curve, the central portion of which may be parallel to the isoelectric line.

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THE HEMOLYTIC EFFECT OF INDOL IN DOGS FED NORMAL DIETS

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(Received for publication, September 22, 1937)

Experiment has shown (1) that anemia of severe degree occurs when indol is fed to dogs maintained on a deficient diet, causative of canine black tongue. The same amount of indol causes either no anemia or a transient and mild anemia in animals taking a normal, mixed diet. Sufficiently large amounts of indol will cause severe anemia, however, even if a normal diet is fed, hence the hypersusceptibility of the animal fed the deficient diet is quantitative rather than absolute.

Three explanations of the anemia-producing effect of indol are possible. (*a*) It is destructive to erythrocytes *per se* and more so when the diet is deficient, or (*b*) it exerts a depressing effect upon the production of erythrocytes by the bone marrow, or (*c*) a destructive action takes place that is uninfluenced by the diet but regeneration is less active in the presence of a poor diet.

To obtain information concerning the first of these possibilities the excretion of bilirubin has been studied in animals fed normal diets with and without the administration of indol. Only in this way could a quantitative measurement of the factor of blood destruction be obtained.

The relation between the rate of excretion of bile pigment and the rate of destruction of erythrocytes has been discussed by Rous (2) and his coworkers as well as by Whipple (3). Whereas the technical methods for the measurement of bile pigment are not sufficiently accurate to allow precise analysis, nevertheless under constant experimental conditions, pronounced and sustained variations in the output of bilirubin can be taken as satisfactory evidence of an increased rate of destruction of blood. According to Broun (4) 1 gm. of bilirubin represents 1 gm. of hematin, and the hemoglobin molecule yields 4

per cent of hematin by weight. For a detailed discussion of the subject the original papers should be consulted.

Methods

Sterile biliary fistulae were prepared according to a somewhat modified technic of Rous and McMaster (5). The common bile duct was sectioned, the fundus of the gall bladder opened *in situ* and a No. 24 catheter sewn in place. The gall bladder was then invaginated over the catheter to make a tight joint. The catheter was led to the pelvis and joined there by a glass U tube to a rubber tube which led out through an oblique incision just below the costal margin. The bile was collected in a rubber balloon which could be drained by a side arm. Strict aseptic precautions were observed whenever the line was opened. The external apparatus was covered by a wicker basket covered by two fitted canvas jackets closed by talon fasteners.

If adequate precautions are taken, sterile bile can be collected for months before infection sets in. The animals eat well and maintain their weight. No animal was employed for experiment in which the bile was infected or in which there was evidence of liver insufficiency as shown by an increased bilirubin content of the blood serum.

The daily output of bilirubin was measured by the method of McMaster (6). To save time, collections of 2 or 3 days were frequently analyzed together. This procedure was justified since only average yields over considerable periods were compared. As can be seen from the charts there exists a moderate day-to-day variation in the yield of bile pigment. These are quite out of the range of the very pronounced changes which are taken as evidence of variation in the rate of hemolysis.

The technic described, though difficult and cumbersome, was selected as the only one which was suitable.

The dogs were kept in metabolism cages to eliminate the factor of exercise which is known to affect the output of bile pigment. The normal diet fed was a uniform mixture of cooked beef, bread, and dog biscuit. Certain experiments were made on animals which were wholly deprived of bile and others in which 50 cc. of dog bile were fed daily. In rare instances beef bile was substituted for dog bile. Furthermore, the feeding of this amount of bile was not reflected in an increased rate of excretion of bile pigment by the fistula, although larger amounts of fed bile have been shown to do so (5). In no instance was a pathologic manifestation referable to deprivation of bile observed.

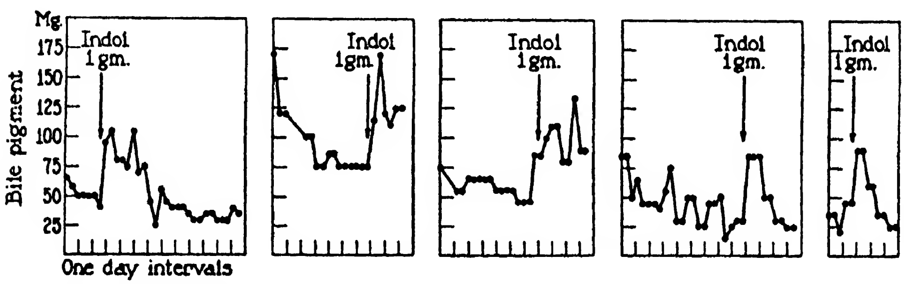
The indol was a commercial product of reasonable purity. It was fed by hand in ordinary absorbable gelatin capsules. The animals were observed carefully to see that the capsules were not vomited. A further check on absorption was at hand in the presence of indican in the urine.

Fistulae between the renal pelvis and the urinary bladder as well as simple determinations of the content of pigment in the stools and urine were discarded as unsatisfactory for these particular experiments.

RESULTS

The changes in the rate of excretion of bilirubin following the administration of indol during the feeding of a normal diet are shown in Text-fig. 1. In all of the experiments shown, a single dose of 1 gm. of indol was fed; a more sustained effect follows the repeated administration of the same amount. Such tests are open to the possible objection, however, that sufficient indol or its metabolic breakdown products are secreted in the bile to give a false colorimetric test for bilirubin

The increased rate of excretion of bilirubin is well defined, is greater than the spontaneous variations, and is sufficiently sustained to rule out technical error resulting from the effect of indol on the colorimetric determinations. Occasional instances are encountered in which no



TEXT-FIG. 1. Effect of the oral administration of indol on the rate of excretion of bilirubin.

increase can be detected and on the other hand rare instances have been encountered in which more marked increases are seen. The charts presented are typical of the average response.

DISCUSSION

From the experiments reported, it is quite apparent that when indol is administered to dogs taking a normal diet a slight and irregular increase of bilirubin output occurs. Since 1 mg. of bilirubin is derived from roughly 0.18 cc. of blood even an increased blood destruction of 9 cc. daily would result in an increased bile pigment output of 50 mg. or double the normal values. The remarkably labile blood volume of the dog and its apparently great reserve of blood in storage depots, makes a loss of this amount of blood perfectly possible without the development of apparent anemia providing a normal diet is fed. The

studies of Robscheit-Robbins, Walden, and Whipple (7) present striking evidence of the amazing regenerative power for blood possessed by the normal organism. Hence an apparent failure of anemia to develop when indol is administered does not necessarily mean that indol is entirely without hemolytic power under normal dietary conditions. It simply indicates that sufficient hemolysis has not taken place to effect a discernible change in the number of circulating formed elements. This factor could of course be controlled by accurate measurements of the blood volume. Two objections to this procedure existed in our experiments. Sufficient blood for repeated blood volume determinations would represent a vastly greater loss than was reflected in the increased output of bilirubin. Secondly the available methods for determinations of blood volume are not accurate within the required limits.

Application of the method of Harrop and Barron (8) to the study of the liver function of the animals with total biliary fistulae indicated that not infrequently a distinct depression of function was present, even though no clinical jaundice was apparent. It is possible that the hemolytic effect of indol administration may reflect a slight though definite hypersusceptibility to that compound associated with the mildly abnormal experimental conditions. Certainly the drug was not as well tolerated as was the case in normal animals without biliary fistulae. Despite the oral administration of fresh dog bile the constant, slow delivery of bile from the common duct which exists normally could not be duplicated in the experiments.

The objection may be advanced that sufficient indol or its derivatives may have been excreted in the bile to give a false reading for bilirubin. Opposed to this is the fact that no indol could be demonstrated in the bile in more than a trace by steam distillation followed by spectroscopic study. Furthermore, it is clear from the figures that the increased output of bilirubin persisted for several days after a single dose of indol. Examination of the blood of the animals showed that normal indican levels were present 8 hours after the administration of 1 gm. of indol by mouth.

CONCLUSION

Indol is mildly hemolytic when fed to dogs taking normal diets.

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INDUCED SUSCEPTIBILITY OF THE BLOOD TO INDOL

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Studies previously reported from this laboratory (1) present evidence that the oral administration of amidopyrine is followed by anemia in dogs fed a deficient diet which is causative of black tongue. Neither amidopyrine alone, in the amounts employed, nor the diet alone, resulted in anemia under the experimental conditions observed. The anemia could be cured, moreover, by supplementing the diet with a factor in which it is deficient, even though the administration of amidopyrine was continued. This phenomenon of an increased susceptibility of the blood to amidopyrine under conditions of dietary deficiency, suggested the existence of a general principle which might also apply to the aromatic compounds produced by endogenous metabolism. Of those compounds, indol has been selected for study since some information is available concerning its metabolism.

Indol has been much studied chemically but relatively little as concerns the pathological effects which result from its administration. Houssay (2) has reported studies of the conjugation of indol and has reviewed the literature on the subject. Houssay concludes that indol is converted into indican by the liver, since that conversion occurs normally after removal of the intestine and after nephrectomy, but not after hepatectomy. Excretion by the kidney is proved by the presence of indican in the urine and by its accumulation in the blood after the removal of both kidneys. Büngeler (3) injected indol into mice and observed that anemia resulted, but since he was concerned only with the development of leukemia, the studies of the erythrocytes are not sufficiently detailed to be analyzed. Furthermore any procedure involving the injection of indol is not applicable, since unconjugated indol is not present in the circulating blood under any but the most abnormal circumstances (Houssay, 2). Certain facts exist, however, which suggest that the aromatic compounds derived from endogenous sources may play some rôle in causing disease. Tönnis and Horster (4) in a series of papers have described the production of indicanuria and anemia in dogs with surgically formed, inactive, open jejunal segments. Relief of anemia as well as the associated symptoms seems to have followed the administration of liver extract in these animals.

Methods

The animals employed were mongrel dogs of about 7 kilos in average weight. They were kept under standard conditions in individual cages with bedding of wood shavings.

The so called normal diet was one which is fed as a routine and empirically is known to be capable of maintaining dogs in good health over a period of several years. It is a mixture of cooked beef, bread, and dog biscuit. The black tongue diet is that described by Goldberger (5). It is known to cause acute black tongue when fed, without supplement, for a period of from 5 to 12 weeks. In an extensive study the feeding of this diet has never been known to cause symptoms in normal dogs after a shorter interval. The corn meal, peas, and casein were mixed and cooked for 2 hours in a steam cooker. The remaining ingredients were then added and thoroughly mixed. The animals were fed daily and were allowed to eat as much as they chose.

Blood was taken from the jugular vein in a standard amount of potassium oxalate for routine examinations. Determinations of the numbers of erythrocytes and leukocytes were made in standard pipettes and counting chambers. The hemoglobin was estimated by the Sahli method, employing a glass standard. The Sahli tubes were carefully calibrated and checked at frequent intervals by the O_2-combining capacity method of Van Slyke.

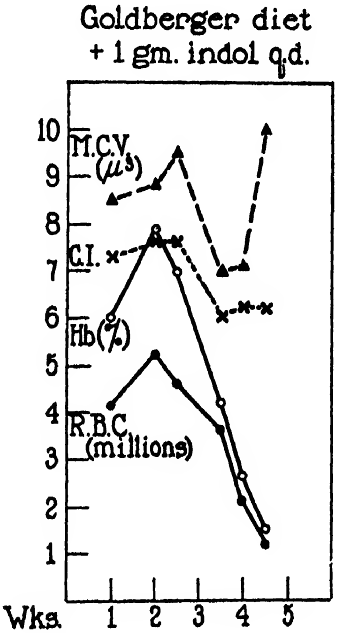
The indol used was a commercial crystalline product of reasonable purity. It was fed in ordinary absorbable capsules which were placed in the back of the animal's pharynx and forced with the finger to a point where they were swallowed. The animals were carefully observed to see that no capsules were regurgitated. The liver extract used was the powdered material (Eli Lilly and Company) of which 4 gm. are derived from 100 gm. of liver. It was made up to a 50 per cent solution in water and fed by stomach tube. Reduced iron (ferrum reductum U.S.P.) was used in a few experiments. It was administered in absorbable capsules containing 1 gm. each. The vegex was the commercial salt autolysate of brewers' yeast made up to a 50 per cent solution in water.

EXPERIMENTAL

In all, 11 different types of experiments were made as follows: Indol was administered: (1) while a normal diet was fed; (2) as a brief experiment to dogs in a state of deficiency following the feeding of the black tongue diet; (3) during periods of deficiency and during subsequent periods when the diets were supplemented with yeast; (4) throughout successive periods of normal and deficient diets; (5) during periods of deficiency and during subsequent periods when the diet was supplemented with liver extract; (6) while an exclusive diet of milk was fed; (7) while a basal diet was fed which is not causative of

black tongue; (8) while the animals voluntarily abstained from food. (9) The effect on liver function of the administration of indol was tested. (10) The effect of splenectomy on the anemia-producing effect of indol was observed. (11) The levels of indol and indican in the blood were observed.

In this communication certain of the experiments are reported together for the sake of brevity.



TEXT-FIG. 1. Dog 1

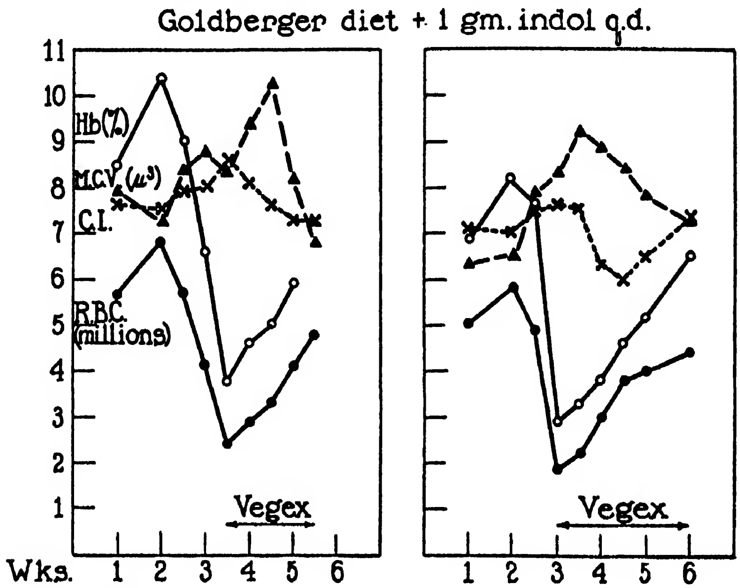
TEXT-FIG. 2. Dog 2

TEXT-FIGS. 1 to 9. Levels of the blood in 9 dogs given 1 gm. of indol daily after deficiency was established.

Acute Hypersusceptibility to Indol

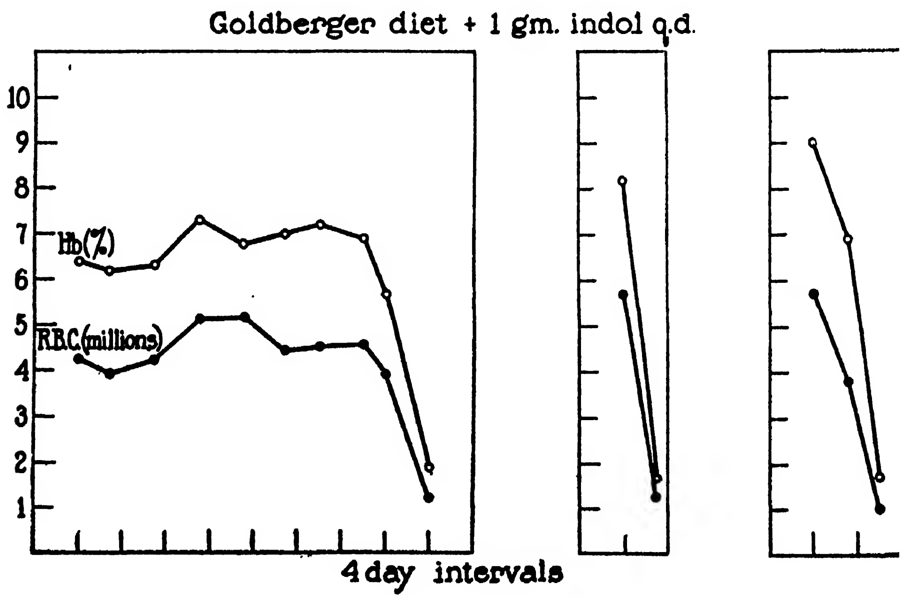
Experiments 1 and 2 (Text-Figs. 1 to 9).—The black tongue diet was fed until the first signs of erythema of the labial mucous membrane appeared. This period varied from 6 to 10 weeks in different experimental animals. Indol was then fed in amounts of roughly 100 mg. per kilo of body weight. A prompt and very marked fall in the numbers of erythrocytes occurred, a decrease from 5,000,000 to something over 1,000,000 cells per mm.^{3} of blood in less than 1 week being common. The anemia was associated with a normal or moderately

elevated leukocyte count and distinctly increased numbers of platelets. The animals became pale and lethargic but continued to take food in most instances. Examination of films of the blood revealed a striking



TEXT-FIG. 3. Dog 3

TEXT-FIG. 4. Dog 4



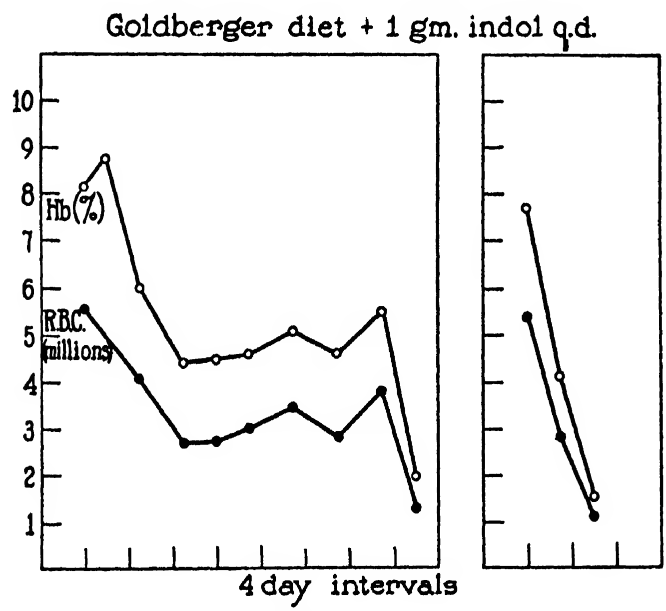
TEXT-FIG. 5. Dog 5

TEXT-FIG. 6
Dog 6

TEXT-FIG. 7
Dog 7

variation in the size and shape of the erythrocytes. If the administration of indol was continued the anemia occasionally terminated fatally but not infrequently an incomplete remission took place, marked by an increase of the circulating reticulocytes to levels between 10 and 20 per cent and an increase in the number of erythrocytes to between 2,000,000 and 3,000,000 per mm.[3]

Control animals taking a normal diet and fed the same amount of indol as those just described showed in most instances no decrease in erythrocyte levels greater than the variations which are normal for the dog. Occasionally a well defined drop in erythrocytes to levels



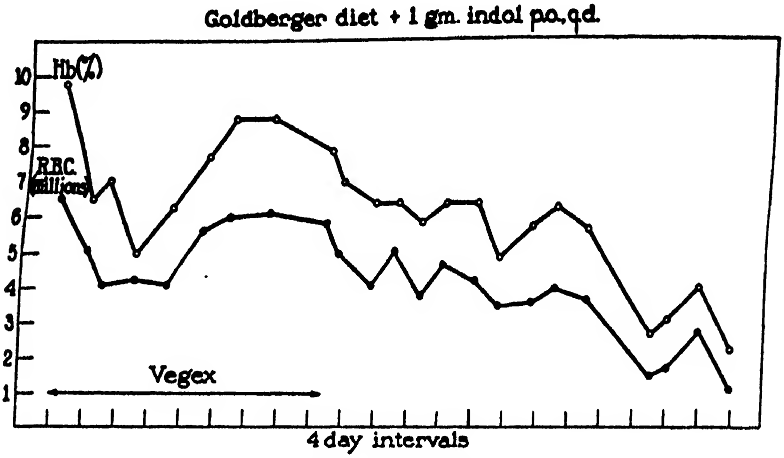
TEXT-FIG. 8. DOG 8 TEXT-FIG. 9. Dog 9

in the 3,000,000 per mm.[3] range occurred, but this was distinctly unusual and was promptly recovered from, although the drug was continued. In no instance did any animal eating the normal diet show a degree of anemia which approached that regularly obtained in the animals fed the deficient diet.

Chronic Hypersusceptibility to Indol and the Treatment of the Anemia with Yeast

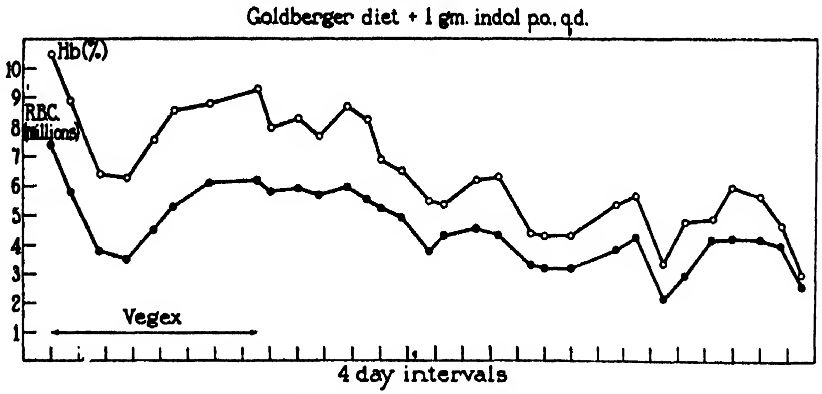
Experiment 3 (*Text-Figs. 10 and 11*).—Previous studies had shown that the symptoms following regularly the feeding of the black tongue

diet without supplement could be prevented by the administration of yeast rich in the vitamin B_2 (G) complex, Goldberger (5). Accordingly the effect of supplementing the diet with a suitable yeast source of that vitamin was tried in animals with anemia resulting from the



TEXT-FIG. 10. Dog 10

TEXT-FIGS. 10 and 11. Levels of the blood in 2 dogs given 1 gm. of indol daily during periods of yeast supplement to the black tongue diet and during subsequent periods of yeast when supplement was omitted.



TEXT-FIG. 11. Dog 11

administration of indol during a state of deficiency. The results are shown in Text-figs. 10 and 11. Following the supplement with yeast a prompt and decided rise in the levels of the blood took place which could be maintained as long as the supplement was given, although the administration of indol was continued throughout the

experiment. When the supplement was discontinued, however, progressive anemia appeared.

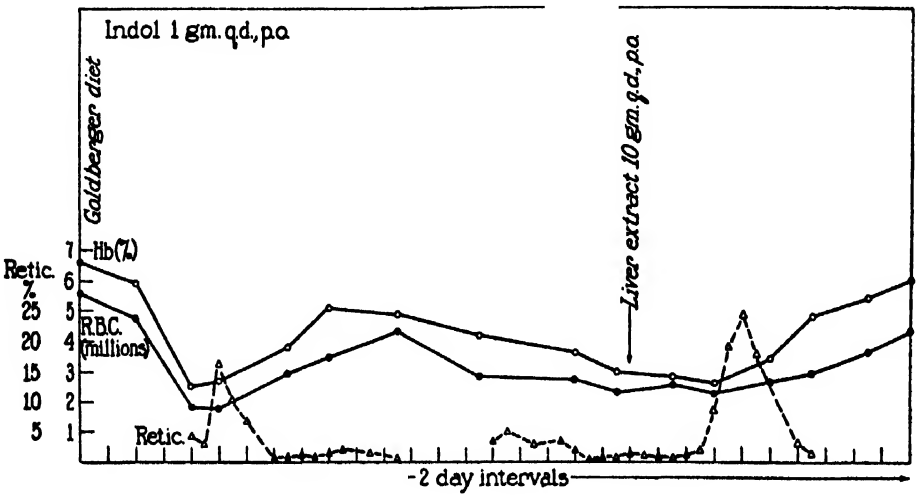
Chronic Hypersusceptibility to Indol and the Treatment of the Anemia with Liver Extract

Experiments 4 and 5 (*Text-Figs. 12 to 18*).—Goldberger (6) has shown that liver extract (Lilly N.N.R.) is preventive of the symptoms of black tongue which resulted from the feeding of the diet used in our experiments. Since the same liver extract is also preventive and curative of pernicious anemia in human beings the effect of its administration was studied in the chronic anemia in the dog resulting from indol and the deficient diet.

In three animals, Nos. 12, 13, and 14, Text-figs. 12, 13, and 14, the black tongue diet was fed until a definite state of deficiency was considered to have been established. Indol was then administered as in Experiment 1 and the anemia promptly developed. Liver extract (Lilly N.N.R.) was then fed by stomach tube in 10 gm. amounts daily. In every instance an increase in the number of circulating erythrocytes took place, associated with an increase in the levels of hemoglobin. The supplement of liver extract was discontinued as soon as approximately normal levels of the blood were obtained, but the feeding of indol as well as the deficient diet was continued.

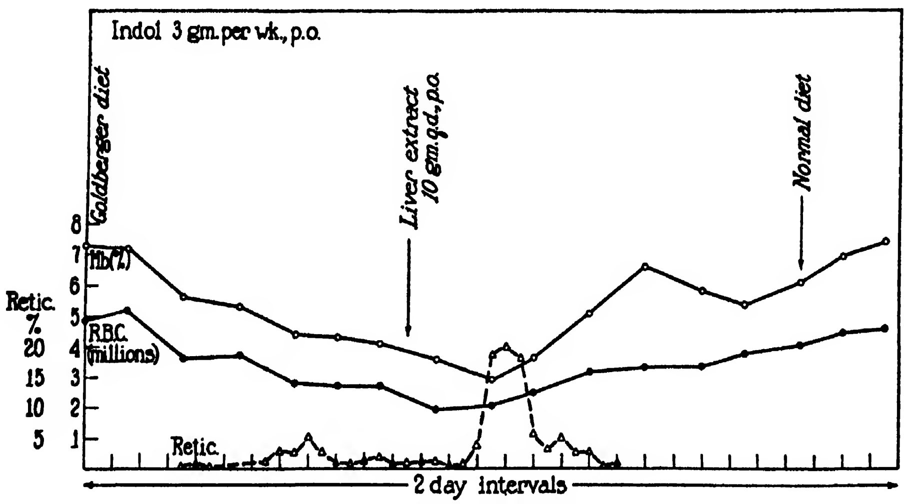
As would be expected from the results of the short experiments, after an interval of from 3 to 5 weeks from the time when the supplement of liver extract was omitted, a slow, progressive decrease in the levels of erythrocytes and hemoglobin appeared and continued until only between 2,000,000 and 2,500,000 erythrocytes per $mm.^3$ were present. This was considered to be a suitably severe form of anemia for test. The levels of reticulocytes varied somewhat during the periods of the anemia. In certain instances they were irregularly elevated for a time and in others no elevation above 2 per cent appeared. In every experiment the reticulocytes were allowed to become stabilized at low levels before therapeutic test. When a severe anemia, with reticulocytes stabilized at a low level, had been obtained, liver extract 10 gm. (Lilly N.N.R.) was administered daily by stomach tube. In every animal a prompt and decided rise of reticulocytes occurred. Levels of from 10 per cent to 75 per cent were obtained within 10 days after

treatment was begun. The number of reticulocytes promptly fell and this was followed by a rise in the levels of erythrocytes and hemo-



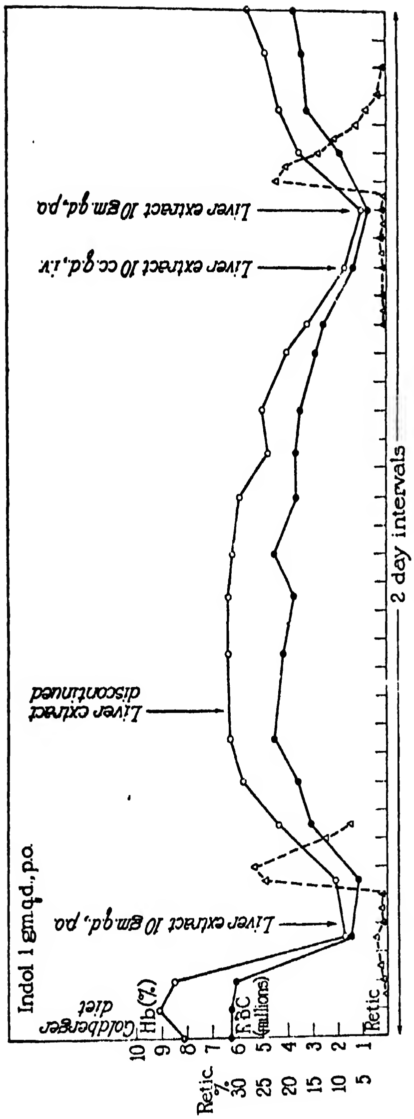
TEXT-FIG. 12. Dog 12

TEXT-FIGS. 12 to 18. Levels of the blood in 7 dogs given indol daily during periods of normal diet, of black tongue diet, and of supplement with liver extract.

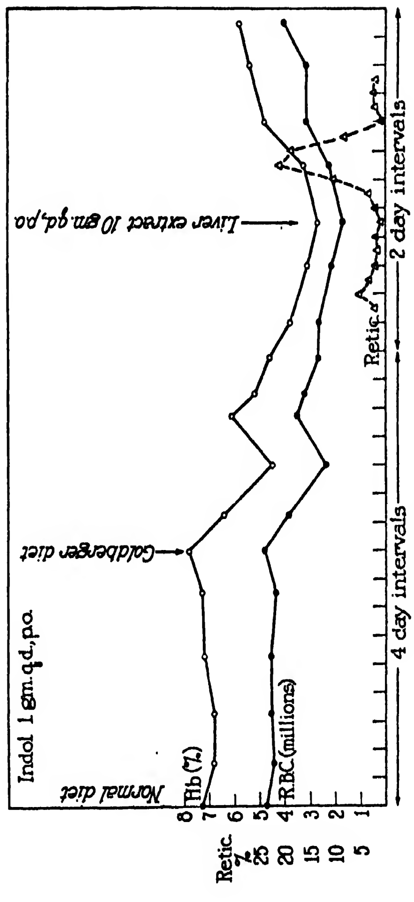


TEXT-FIG. 13. Dog 13

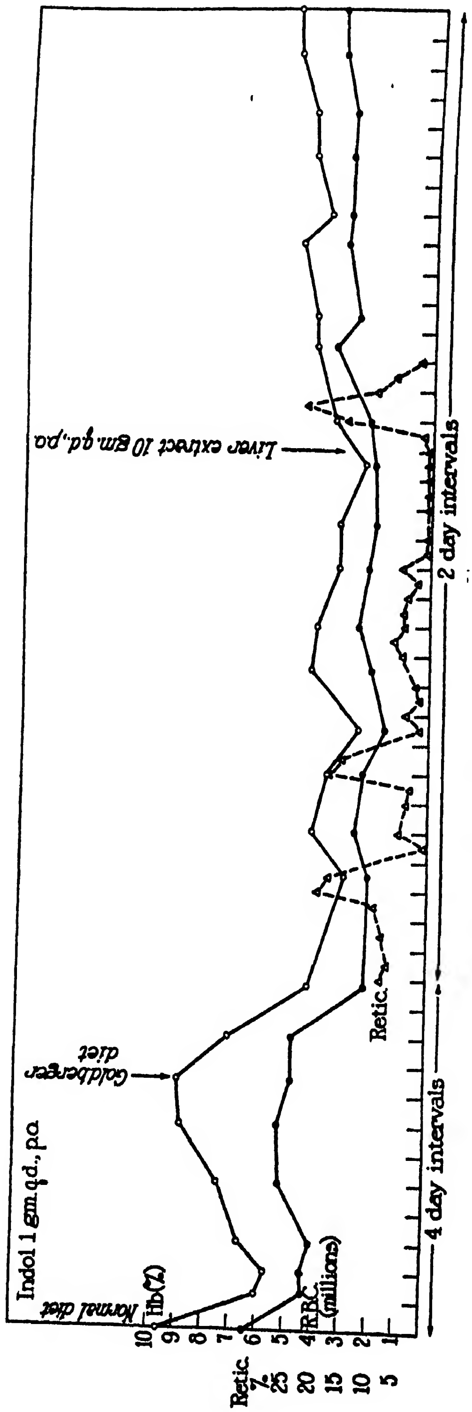
globin. Simple supplement of the diet with liver extract was not sufficient to restore absolutely normal values for the blood in every experiment, and where this was the case a normal diet was substituted



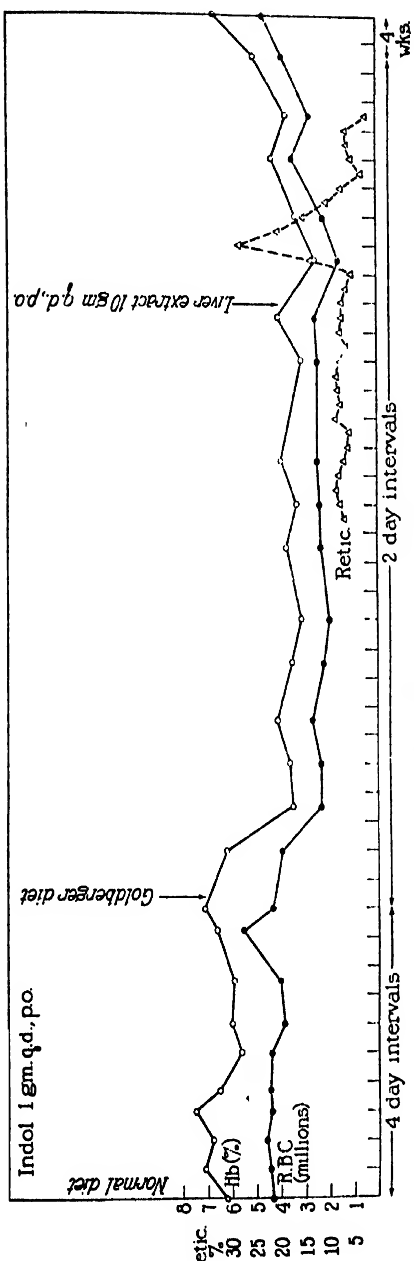
TEXT-FIG. 14. Dog 14



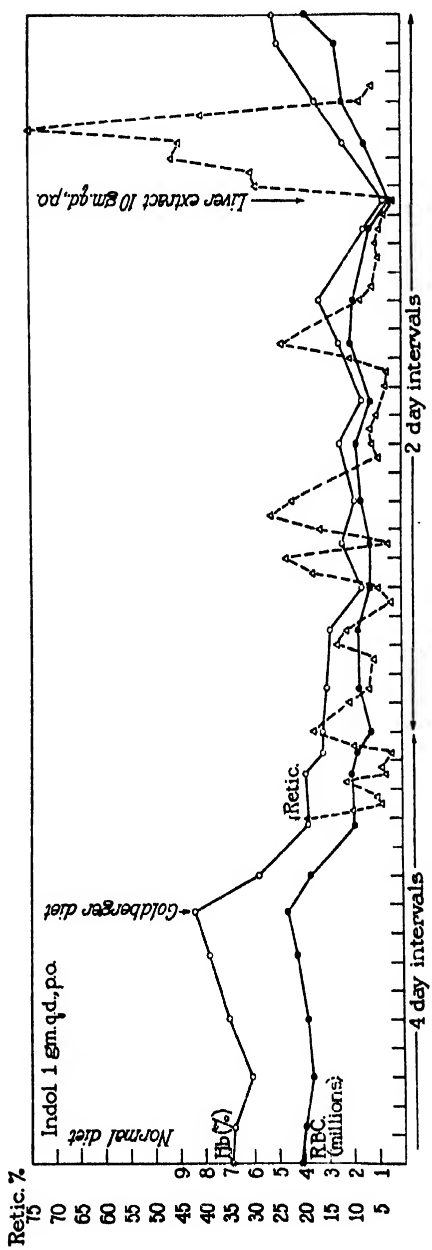
TEXT-FIG. 15. Dog 15



TEXT-FIG. 16. Dog 16



TEXT-FIG. 17. Dog 17



TEXT-FIG. 18. Dog 18

for the deficient one. This move effected complete cure of anemia in every instance despite the continued feeding of indol.

In the greater number of the observations, Nos. 15 to 18, a control period was observed, during which the normal diet and a standard amount of indol were fed. In some instances, as in animals 15 and 17 there was no fall of blood levels but occasionally, as in observations 16 and 18 a mild decrease of erythrocytes and hemoglobin occurred which promptly disappeared as soon as sufficient time had elapsed to allow the normal hematopoietic mechanism to accommodate for the increased demand put upon it by the administration of indol. After the normal diet had been fed sufficiently long to establish the fact that the indol was tolerated without anemia, the black tongue diet was substituted and the administration of indol was continued. As in the experiments just described a gradual fall of the numbers of erythrocytes and of hemoglobin resulted until a severe degree of anemia was established. As before, a considerable variation in the numbers of reticulocytes was observed in the first part of the period of anemia but in due time they became stabilized at low levels. The institution of therapy with liver extract, other factors remaining unchanged, was followed by the expected reticulocyte peak and by an increase of blood values to levels which approached the normal. A striking feature of the response to the administration of liver extract was an increase in the number of leukocytes, although they had not been notably few during the severe phase of anemia. This increase usually preceded the increase in erythrocyte numbers by several days and coincided with the appearance of elevated levels of reticulocytes.

The histological changes in the bone marrow will be described in a separate communication. It suffices to state here that biopsies of femoral marrow, removed during a control period before the experiment was begun, showed a normal distribution and the expected orderly maturation of cells with all stages of development represented. Specimens of femoral marrow removed by biopsy and at autopsy at the height of the anemia showed a very marked increase in the cellularity of the marrow with a lack of mature forms, both of the red and white cell series. Biopsies of marrow after weeks of the administration of indol with a normal diet showed no significant variation from normal.

Protocols of Experiments 4 and 5.—

Animal 12.—

Dec. 22. Goldberger diet begun. Weight 17.5 kilos.

Jan. 22. Indol feeding begun, 6 gm. weekly. R.B.C. 5,500,000. Hb 66 per cent. W.B.C. 15,000.

Jan. 29. Very pale and weak. No stomatitis. R.B.C. 1,830,000. Hb 25 per cent. W.B.C. 51,500. Reticulocytes 4.2 per cent.

Jan. 31. General condition improved. R.B.C. 1,760,000. Hb 27 per cent. W.B.C. 25,200. Retic. 16.2 per cent. Despite the continuation of the feeding of the diet and the administration of indol a mild remission occurred, presumably explained by the extension of active, hematopoietic marrow.

Feb. 13. General condition excellent. R.B.C. 4,330,000. Hb 48 per cent. W.B.C. 10,000. Retic. 0.8 per cent. From this point a slow fall of the levels of the blood took place, possibly because no further extension of hematopoiesis was possible under the experimental conditions.

Mar. 1. Animal very pale and weak. No stomatitis. R.B.C. 2,360,000. Hb 30 per cent. W.B.C. 10,500. Retic. 0.8 per cent. Therapy with liver extract begun, 10 gm. daily, by mouth.

Mar. 8. General condition improved. R.B.C. 2,229,000. Hb 26 per cent. W.B.C. 74,800. Retic. 8.0 per cent. The marked elevation of white count and beginning of a rise of reticulocytes indicates the onset of remission.

Mar. 15. General condition excellent. R.B.C. 2,910,000. Hb 48 per cent. W.B.C. 19,500. Retic. 1.4 per cent. The rise of reticulocytes has subsided and an increase of levels of the blood is in progress.

May 9. General condition excellent. R.B.C. 5,150,000. Hb 82 per cent. W.B.C. 20,500.

Animal 13.—

Jan. 25. Feeding of Goldberger diet begun. Weight 8.6 kilos. Administration of indol begun, 3 gm. weekly. R.B.C. 5,140,000. Hb 72 per cent. W.B.C. 10,300. Retic. 0.4 per cent.

Feb. 15. Pale and weak but eating well. R.B.C. 1,990,000. Hb 36 per cent. W.B.C. 11,600. Retic. 1.2 per cent. Treatment with liver extract, 10 gm. daily by mouth, was begun.

Feb. 19. General condition slightly improved. R.B.C. 2,050,000. Hb 29 per cent. W.B.C. 42,600. Retic. 18.8 per cent. The elevated leukocyte and reticulocyte counts indicate the impending remission.

Mar. 1. General condition excellent. R.B.C. 3,370,000. Hb 66 per cent. W.B.C. 13,000. Retic. 1.4 per cent. The numbers of leukocytes and reticulocytes have become normal and the increased levels of the blood are clearly in evidence.

Mar. 12. Normal diet substituted for the Goldberger diet. R.B.C. 4,000,000. Hb 61 per cent. W.B.C. 13,300.

Mar. 19. Condition excellent. Experiment terminated. R.B.C. 4,560,000.

Hb 74 per cent. W.B.C. 12,100. In a second experiment with similar course this animal was destroyed at the height of the anemia and the bone marrow subjected to histological study.

Animal 14.—

Nov. 30. Feeding of Goldberger diet begun. Weight 10.5 kilos.

Jan. 2. Administration of indol begun, 6 gm. weekly. Animal in excellent condition. R.B.C. 6,250,000. Hb 91 per cent. W.B.C. 11,500. Retic. 0.4 per cent.

Jan. 7. Animal very pale and weak; slight erosion of labial mucous membranes. Eating diet. R.B.C. 1,430,000. Hb 17 per cent. W.B.C. 23,100. Retic. 2.2 per cent. Supplement of liver extract, 10 gm. daily, by mouth begun.

Jan. 11. Animal in poor condition; not eating the diet. R.B.C. 1,150,000. Hb 21 per cent. W.B.C. 76,200. Retic. 24.6 per cent. This was the first day of the change in the number of leukocytes and reticulocytes as an indication of the beginning of the induced remission.

Jan. 23. Animal in excellent condition. Eating well. Liver extract discontinued. R.B.C. 4,540,000. Hb 63 per cent. W.B.C. 10,000. Retic. 0.8 per cent. From this point on there was a prolonged slow drop in the levels of the blood.

Feb. 8. Superficial early erosions of the labial mucous membranes have appeared. R.B.C. 3,680,000. Hb 58 per cent. W.B.C. 11,900. Retic. 2.4 per cent.

Feb. 25. Ulcerated lesions of the tongue and cheeks are well defined. Animal weak and pale. Not eating. R.B.C. 1,370,000. Hb 17 per cent. W.B.C. 21,800. Retic. 1.2 per cent. Liver extract, 10 cc. intravenously, daily.

Mar. 1. Animal very weak and pale. R.B.C. 750,000. Hb 10 per cent. W.B.C. 139,900. Retic. 0.8 per cent. The anemia was so severe that it was supposed that the animal's life was in danger in spite of the intravenously administered liver extract. Actually in light of subsequent observations the rising leukocyte count and the drop of general blood levels should have been taken as an indication of the onset of remission. Therapy was changed to orally administered liver extract however.

Mar. 3. The reticulocyte count has risen today to 22.8 per cent and the animal is markedly improved. Since the peak of reticulocytes has occurred only 48 hours after the institution of oral therapy, it is quite clear that it is in reality due to the parenterally administered substance.

Mar. 5. General condition excellent. Animal eating well. R.B.C. 1,880,000. Hb 35 per cent. W.B.C. 6,800. Retic. 13.6 per cent.

Mar. 12. Animal apparently well. R.B.C. 3,360,000. Hb 48 per cent. W.B.C. 10,900. Retic. 0.4 per cent. The peak of reticulocytes is well past and a clear remission of the anemia is in progress.

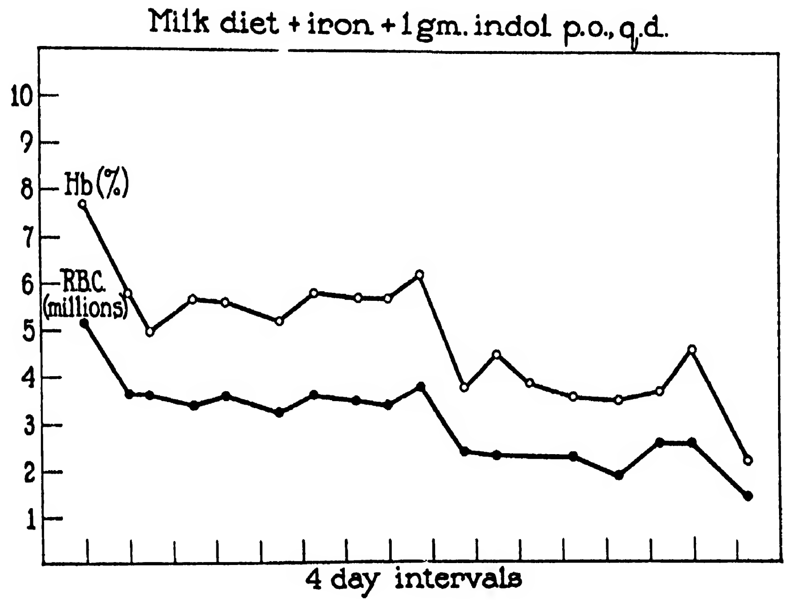
Apr. 30. General condition excellent. R.B.C. 3,860,000. Hb 53 per cent. W.B.C. 11,400. As was so frequently the case in these studies, liver extract,

though completely effective in causing remission, rarely enabled the animal to attain a completely normal level of the blood. Accordingly a normal diet was substituted for the Goldberger diet, the administration of indol being continued.

May 21. Normal levels of blood have been obtained. Experiment discontinued. R.B.C. 5,150,000. Hb 88 per cent. W.B.C. 11,000.

Hypersusceptibility to Indol Resulting from Diets of Milk

Experiment 6 (*Text-Figs. 19 to 21*).—All the studies of deficiency disease which have involved the use of the Goldberger diet causing

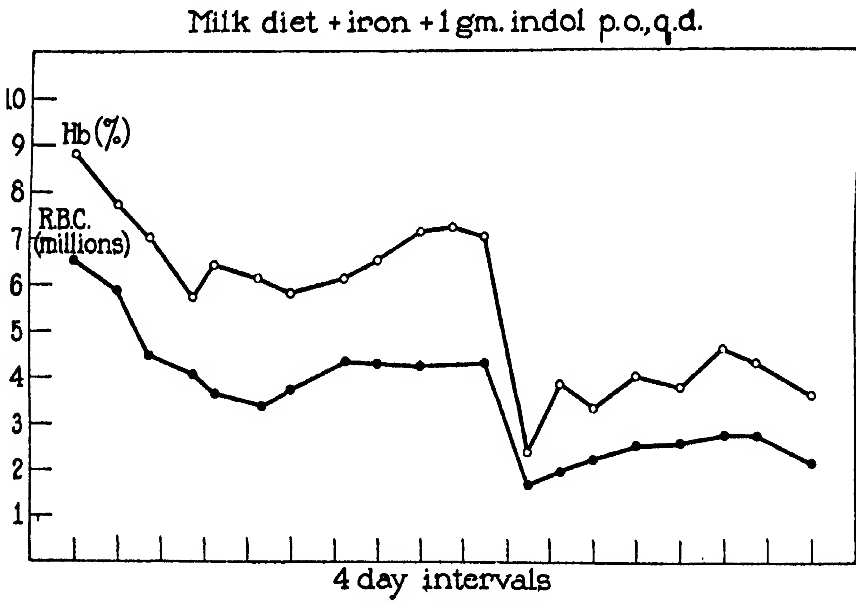


TEXT-FIG. 19. Dog 19

TEXT-FIGS. 19 to 21. Levels of the blood in 3 dogs given indol daily while fed a diet of milk with and without supplement by liver extract.

black tongue have been open to certain objections. Among them is the fact that the clinical syndrome of black tongue does not occur when the simple diet of Cowgill, which lacks the heat stable fraction of the vitamin B complex, is fed (Zimmerman, 7). Moreover, the Goldberger diet is exceedingly high in its content of corn and throughout the literature of pellagra there runs a strong suggestion that corn has some peculiarly specific action in causing stomatitis and central nervous system lesions. Rhoads and Miller (8) have shown that the Goldberger diet is not free of the heat stable component of the vitamin

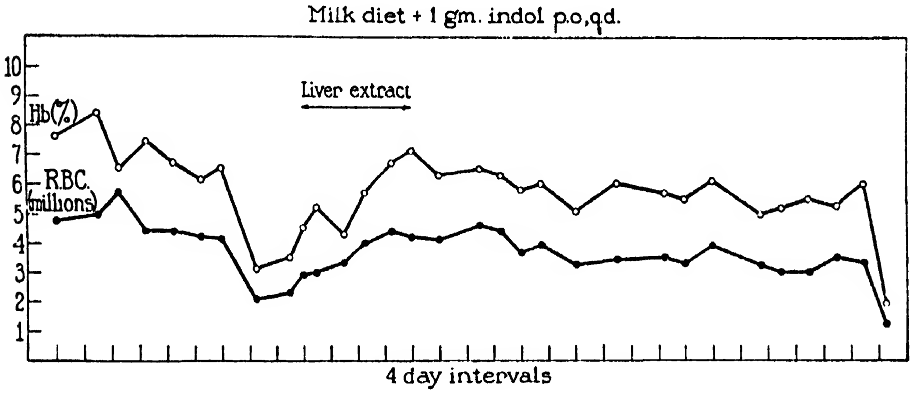
B complex which is required for growth in rats, since rats fed that diet grow at a normal rate. Certain other species, notably guinea pigs and swine, however, will not survive when fed the diet unless it is supplemented with liver extract (9, 10). Evidence has been advanced recently by Birch, György, and Harris (11) as well as by Street (12) that black tongue can be produced by feeding simple basal casein, fat, and glucose diets supplemented with crystalline vitamin B_1 and pure flavin (B_2). In view of the slight doubt concerning the cause of the peculiar syndrome which follows the feeding of the Goldberger



TEXT-FIG. 20. Dog 20

diet, however, it was necessary to control the experiments which have been described by combining the administration of indol with some diet which contained no corn. In a series of unrelated experiments dogs were fed a diet composed exclusively of milk (200 cc. per kilo of body weight daily). To our surprise the animals developed characteristic stomatitis of black tongue and died after a period of about 2 months. Here then was a diet which was known to be rich in flavin, and which was presumed to be rich in the remainder of the vitamin B_2 complex, but which caused black tongue. The objections which could be raised to the experiments on corn diets could be avoided by

feeding milk. Furthermore, although chronic black tongue, produced by treating insufficiently the acute phase of the disease, will result in anemia irregularly, the feeding of milk alone to adult dogs has never done so in our experience. Accordingly a series of animals were fed an exclusive diet of milk and were given the usual dose of 100 mg. per kilo of indol daily, reduced iron 1 gm. daily was administered as a supplement (Text-figs. 19 to 21). Exactly the same anemia occurred as was the case when the Goldberger diet was fed. Severe anemia developed which could be cured by feeding a normal diet or by supplementing the diet of milk by liver extract even though the administration of indol was continued.



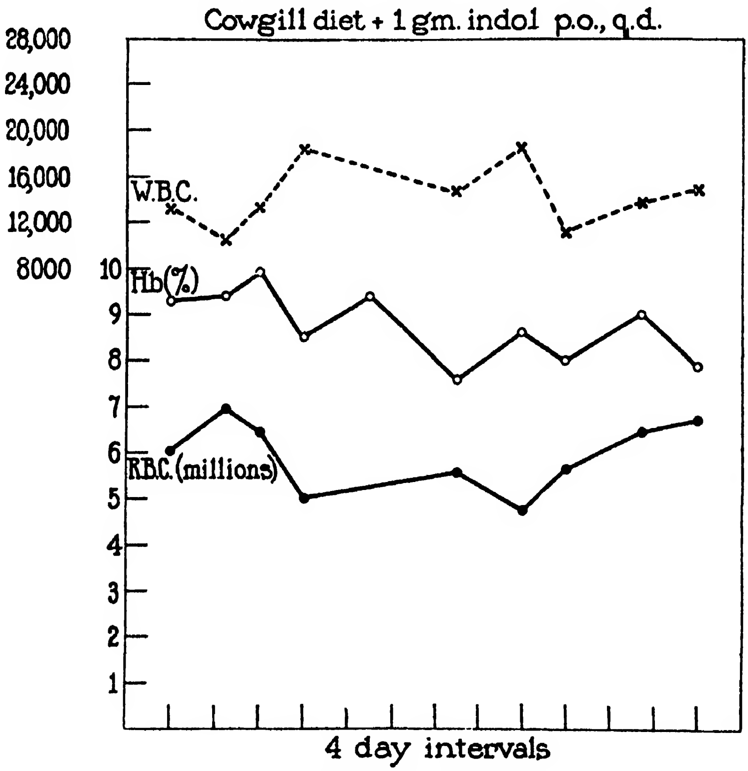
TEXT-FIG. 21. Dog 21

Failure to Induce a Susceptibility of the Blood to Indol by Feeding a Basal Diet

Experiment 7 (*Text-Figs. 22 and 23*).—To control further the dietary factors involved in the experiments, animals were fed the basal diet described by Cowgill (7), and indol was administered at the same time. Both factors of the vitamin B complex were omitted from the diet in the hope that it would be possible to establish the susceptibility of the blood to indol as being due to a lack of some part of that complex. The results were somewhat irregular but in general the indol was tolerated without severe anemia, quite contrary to the absolutely uniform anemia obtained by feeding indol with a Goldberger diet (Text-figs. 22 and 23).

This result was unexpected to some extent and no well substantiated

explanation can be advanced at this time. It is possible, however, that the very high content of casein of this diet may furnish a sufficient amount of the anti-black tongue factor to prevent the susceptibility to indol. It is striking that although the diet is supposedly a basal ration it never gives rise to the symptoms of black tongue. Further studies of this question are in progress, since possibly the rations of Birch, György, and Harris (11), or of Street (12), containing less



TEXT-FIG. 22. Dog 22

TEXT-FIGS. 22 and 23. Levels of the blood in 2 dogs given indol daily while fed the basal ration of Cowgill.

casein and causing black tongue would cause the hypersusceptibility to develop.

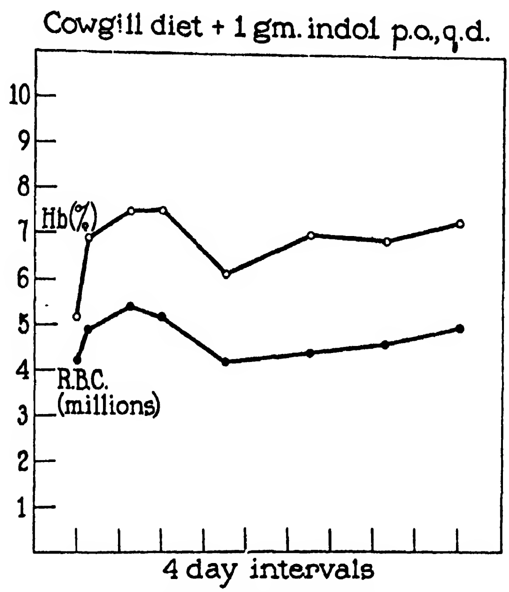
Susceptibility of the Blood to Indol Following Voluntary Abstention from Food

Experiment 8 (*Text-Fig. 24*).—The effect of dietary on the tolerance of the blood to indol is well shown in this experiment. Fig. 24 shows

the course of the anemia in one such animal. Although the black tongue diet was offered each day the food was completely refused. A severe anemia developed promptly, in sharp contrast to the result following the administration of the same amount of indol when a normal diet is taken.

Liver Function Not Affected by Indol

Experiment 9.—Experiments have been published by Miller and Rhoads (13) which show that liver function, as measured by the power of that organ to excrete bilirubin intravenously injected, decreases in

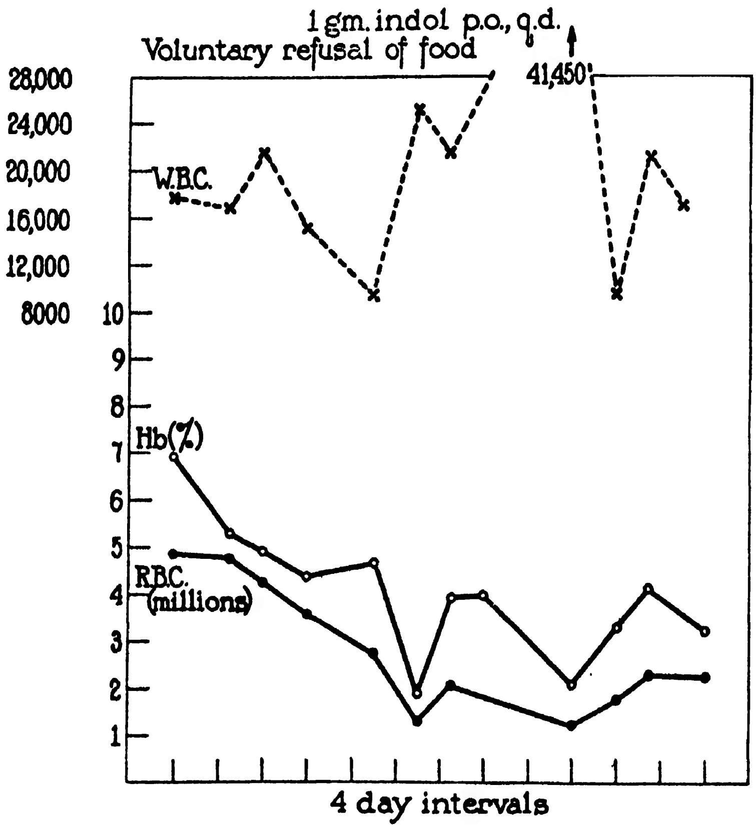


TEXT-FIG. 23. Dog 23

the deficiency which results from feeding the black tongue diet. This finding of deranged liver function is in accord, furthermore, with the beneficial effect of therapy with liver extract. It is required to show, however, in a control experiment that indol *per se* does no damage to liver function. Accordingly the experiment was made of administering 1 gm. of indol daily to 2 dogs taking a normal diet and following the power of the livers to excrete bilirubin. No change was seen in this function over a period of 1 month and no anemia developed. It was concluded that indol did not injure the hepatic function under the experimental conditions observed.

The Effect of Splenectomy

Experiment 10.—In view of the therapeutic effect of splenectomy on certain hemolytic anemias in human beings it was of importance to ascertain whether or not the operation would prevent the development of indol anemia. Accordingly splenectomy was performed in 2 dogs and complete recovery was allowed to occur. The deficient diet was



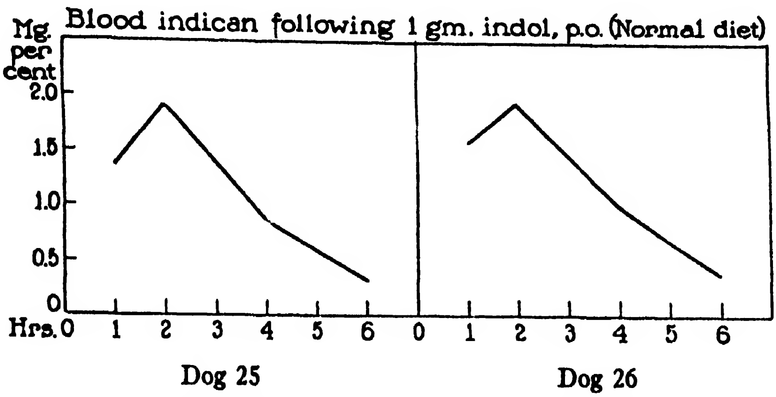
TEXT-FIG. 24. Dog. 24. Levels of the blood in a dog given indol while voluntarily abstaining from food.

then fed and after 4 weeks indol was administered. Anemia of the same degree as that seen in the non-splenectomized animals appeared at the usual time.

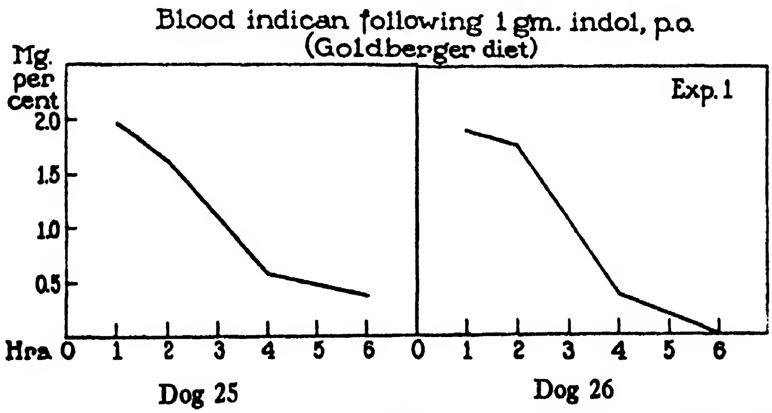
The Levels of Indol and Indican in the Blood

Experiment 11 (Text-Figs. 25 and 26).—The pronounced difference observed between the susceptibilities to indol of normal and deficient

animals suggested that the deficient state resulted either in some change of the rate of absorption of indol from the intestinal tract or of its conversion to indican. These possibilities were subjected to experimental test. By the method of Sharlit (14) the levels of indican in the blood following the oral administration of 1 gm. of indol were ascer-



TEXT-FIG. 25. Levels of indican in the blood at hourly intervals after the oral administration of 1 gm. of indol during the feeding of a normal diet.



TEXT-FIG. 26. Levels of indican in the blood at hourly intervals after the oral administration of 1 gm. of indol during the feeding of a black tongue diet.

tained in two dogs fed a normal diet, and in the same 2 animals after a well defined state of deficiency had resulted from feeding the black tongue diet. As shown in Text-figs. 25 and 26 there was no change detectable between the two nutritional states. Furthermore the levels of indol in the blood were ascertained by the method of Mazzocco (15) in parallel with the levels of indican following both the oral and intra-

venous administration of indol. No difference could be seen between the normally fed animals and those on deficient diets.

DISCUSSION

Certain factors in the experiments which have been described warrant discussion. As seen in the text-figures, the feeding of indol to dogs on a normal diet does result occasionally in a mild anemia which promptly disappears. Histological studies of the bone marrow during this phase of preliminary slight anemia show normal maturation of erythropoietic cells and some increase in number. It is quite apparent that indol feeding is a strain, though a mild one, on hematopoiesis in normally fed dogs and that it is readily compensated for by an increase of functional marrow. When the deficient diet is fed, however, a wholly different picture results from the administration of the same quantity of indol.

The rapid and profound drop in red cells strongly suggests an actual destructive process. In view of the known life of the red cell, anemia would develop much more gradually were only maturation interfered with. Proof of the hemolytic nature of the process will be presented in a subsequent communication.

From the histological appearance of the marrow it seems clear that a great increase in the number of immature marrow cells is a feature at the height of the anemia. Only two possibilities exist to explain this change: (*a*) that the deficient diet lacks a factor which is required for the maturation of erythropoietic cells, and (*b*) that all cells beyond the stage of erythroblast and myeloblast are destroyed in a hemolytic process which is stopped by something contained in liver extract. The early rises of reticulocytes indicate that the marrow can manufacture young cells, or, if the pure hemolytic theory be accepted, that not all reticulocytes are hemolyzed. According to Dock (16) an increase of reticulocytes is as much a feature of cessation of hemolysis as it is of the supplying of a specific maturation factor. From the evidence at hand the final answer cannot be obtained; subsequent communications will deal with this question in greater detail. It is entirely possible, indeed probable, that both processes play some rôle.

Sufficient information is not available from these experiments to discuss the question of oral *versus* parenteral therapy. Suggestive

evidence is at hand from Experiment 3 which favors the view that liver extract parenterally administered is effective. From the studies of Richter, Ivy, and Meyer (17) it is clear that the dog's stomach has little anti-anemia potency for the human being, and this is also true for the liver of the dog. Castle (18) and his coworkers have shown that the anti-anemia principle in liver extract is dependent upon the interaction of a dietary constituent of normal gastric juice.

Since the dog has neither the gastric nor the liver factor we infer that in hematopoiesis it uses the dietary factor as such without the interaction with the gastric factor which is required in the human being. Helmer, Fouts, and Zerfas (19) have shown that liver extract is rich in the dietary factor and it is our belief that in the dosage employed this factor is the effective one in the dog experiments. Until more purified fractions for the treatment of pernicious anemia in the human being are available it seems unwise to continue experiments with liver extract parenterally injected. The results may always be open to the objection that sufficient dietary factor is present to produce an effect.

An analogy inevitably suggests itself between the experimental disease just described and pernicious anemia in the human being. The points of similarity are striking: mucous membrane lesions, gastro-intestinal disturbances, characteristic changes in the morphology of the erythrocytes, absence of hemorrhagic phenomena, low reticulocytes, and a characteristic response to the administration of liver extract. Certain well defined differences are also apparent. Macrocytosis is not a feature since as many or more microcytes are present in the smear than are macrocytes. No serious degree of indolemia or indicanuria can be demonstrated in pernicious anemia and although suppression of maturation in the bone marrow is a feature in the human being, the cell type which predominates in the marrow in these experiments on dogs cannot be proved to be the cell which is predominant in pernicious anemia. No claim is made that the experimental disease has any connection with any disease state of human beings.

The objection will be advanced to these experiments that in the studies reported by Rhoads and Miller (20) the occurrence of anemia in chronic black tongue produced by feeding a Goldberger diet alone has been described. In that publication it is specifically stated that

only after a prolonged chronic disease has been produced by treating insufficiently the acute phase does any anemia occur. Even then it occurs irregularly and is of mild degree. At no time has severe anemia been observed in this laboratory in experiments on a large number of animals during *acute* black tongue. Furthermore in the experiments presented here the controls with milk diets and the voluntary refusal of food rule out any specific effect of the black tongue diet, other than the deficiency.

It appears that under particular dietary circumstances the feeding of indol to dogs produces an effect which is not apparent when a normal diet is fed. Furthermore the effect may be caused to disappear by supplementing the diet with the factor in which it is deficient. Since neither the mode of action of the Goldberger diet, nor the active constituent of liver extract are known exactly it would be idle to speculate at this time concerning the exact mechanism by which the anemia is produced.

CONCLUSIONS

1. Indol, orally administered, causes anemia when certain deficient diets are fed.
2. The same amount of indol causes no considerable hematologic disturbance when normal diets are fed.
3. The anemia can be cured by supplementing the diet with liver extract, or by substituting a normal diet for the deficient diet.
4. Neither the diet alone nor the administration of indol alone produces marked anemia under the experimental conditions observed.

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THE INCREASED SUSCEPTIBILITY TO HEMOLYSIS BY INDOL IN DOGS FED DEFICIENT DIETS

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Evidence has been presented in previous communications (1, 2) that indol, orally administered in suitable amounts, is hemolytic in dogs and that when the animals are fed deficient diets anemia results. The same amount of indol fed to dogs taking a normal diet has little or no effect in causing anemia. Since the hemolytic effect of indol has been proved, two possible ways exist in which anemia might result when the deficient diet is fed: (*a*) the hemolytic effect of the indol may be more marked in the presence of a deficiency or (*b*) the hemolytic effect may be constant and the bone marrow may be less capable of forming erythrocytes when the diet is unsuitable. To settle this question prolonged studies of the excretion of bile pigment and of the erythrocyte and hemoglobin levels in dogs receiving indol and fed normal diets, deficient diets, and deficient diets supplemented by the lacking factor have been made.

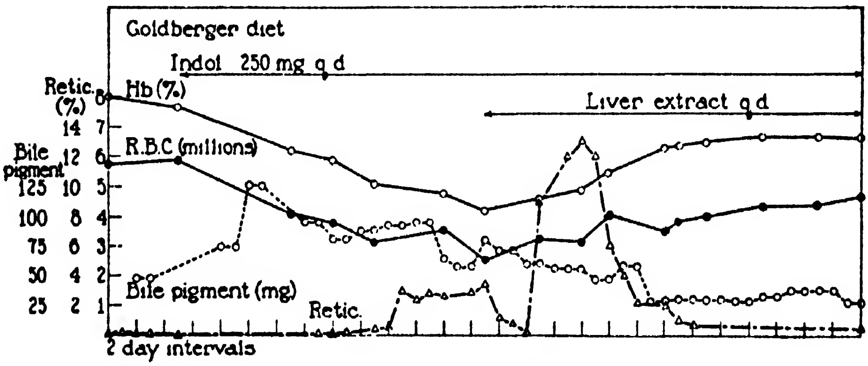
Methods

The technic employed for collecting the total bile in a sterile condition has been described (1, 2). It is a modification of that developed by Rous and McMaster (3). The measurements of the content of bilirubin in the bile have been made by the method of McMaster, Brown, and Rous (4). Although the method is open to certain theoretical objections the experimental results are valid since the interpretation depends upon the comparative values over different periods. The normal diet fed is a mixture of cooked beef, bread, and dog biscuit known empirically to be adequate in its content of vitamins. The Goldberger diet has been described repeatedly in previous publications (5), and is known to produce black tongue regularly in from 5 to 12 weeks. Since that effect can be prevented uniformly by feeding autoclaved yeast and other substances which are rich in their content of vitamin B_2 (G) and cannot be prevented by flavin (Koehn and Elvehjem, 6), its effect is supposed to be due to a lack of a part of the vitamin B_2 (G) complex other than the rat acrodynia factor or flavin.

Fresh dog bile was refed in 50 cc. amounts twice weekly to all the animals. Blood was taken at regular intervals from the jugular vein in standard amounts of potassium oxalate. Counts were made using standardized pipettes and counting chambers. The hemoglobin was estimated by the Sahli method, using calibrated tubes. The indol was a commercial crystalline product. It was fed by hand in ordinary absorbable capsules. All dogs were dewormed with hexylresorcinol and castor oil 1 month before they were put on experiment. The liver extract was Lilly (N.N.R.) powder, 4 gm. of which are derived from 100 gm. of liver. It was made up with water to a 25 per cent solution. In most instances the animals took it avidly.

EXPERIMENTAL

Experiment 1 (Text-Fig. 1).—This animal was fed the deficient diet for 4 weeks before the biliary fistula was formed. The output of bilirubin was allowed to



TEXT-FIG. 1. Dog 1

TEXT-FIGS. 1 to 7. Levels of reticulocytes, erythrocytes, hemoglobin, and daily excretion of bilirubin in dogs fed indol during periods of good and bad diets.

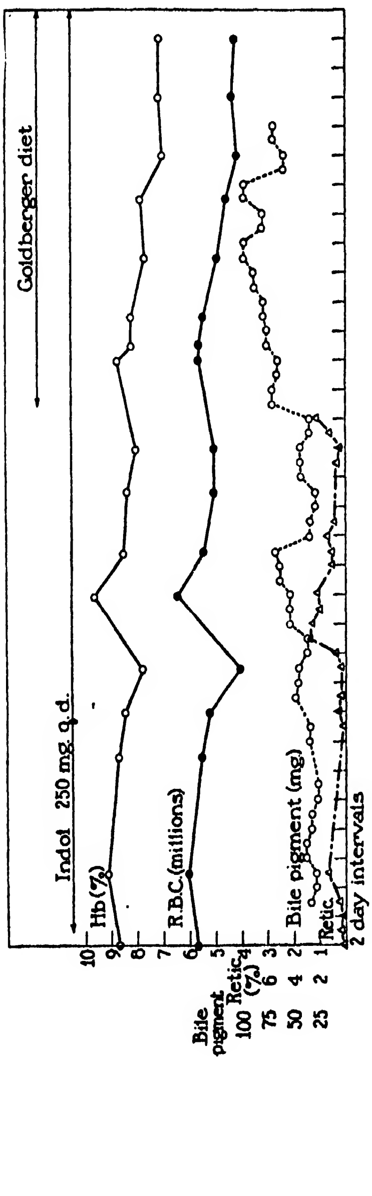
stabilize at about 50 mg. daily before indol was fed. Throughout the period depicted in the text-figure 250 mg. of indol were fed daily. 4 days after the first indol a daily output of bilirubin of 126 mg. was obtained which decreased slightly thereafter to stabilize at a figure between 80 and 90 mg. A progressive decrease in the blood levels from 5,780,000 erythrocytes and 80 per cent hemoglobin to 2,500,000 erythrocytes and 42 per cent hemoglobin took place without any significant elevation of reticulocytes. Over an 18 day period 1,568 mg. of bilirubin were excreted, an average of 87 mg. per day. The diet was then supplemented with 5 gm. of liver extract daily. On the 4th day the reticulocytes increased to 9 per cent and on the 7th day to 13 per cent followed by a progressively rising erythrocyte count to 4,360,000 and hemoglobin values to 63 per cent. Nothing was changed except for the addition of liver extract. The rate of excretion of bilirubin dropped sharply to 50 mg. daily, concurrently with the rise in numbers of reticulocytes, and then continued to decrease slowly. In the 18 days of treatment

859 mg. of bilirubin were excreted, an average of 47 mg. per day, a reduction of nearly 50 per cent of the untreated levels.

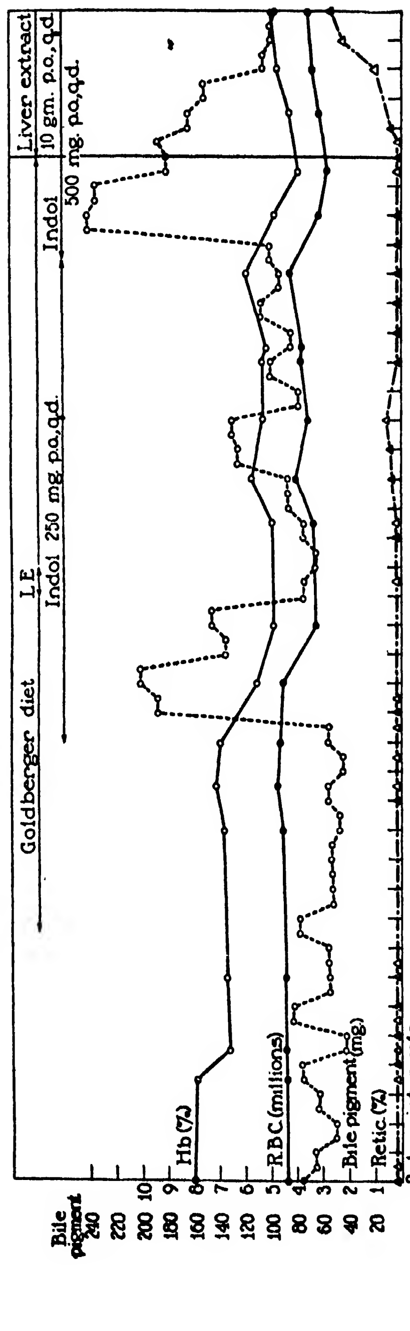
Experiment 2 (Text-Fig. 2).—In contrast to Experiment 1, indol was administered first to this animal while a normal diet was taken. As shown in Text-fig. 2 there was no increase in the excretion of bilirubin following this amount of indol, although the animal was observed for a control period of 33 days. No change in the levels of the blood or the reticulocytes took place. For the 15 days before the diet was changed 635 mg. of bilirubin were excreted, an average of 42 per day. The diet was then changed to that causing black tongue and a remarkably prompt and sustained increase in the excretion of bilirubin became evident. During the 15 days after the change 1,213 mg. of bilirubin were excreted, an average of 80 mg. daily or about double the levels of the control period. Concurrently there was a progressive drop in the blood values from 5,630,000 erythrocytes and 87 per cent hemoglobin to 4,100,000 erythrocytes and 70 per cent hemoglobin. At this point the bile became infected and the experiment was discontinued.

Experiment 3 (Text-Fig. 3).—The black tongue diet was fed for 14 days during which the excretion of bilirubin was extremely constant and the blood levels were stable. The total excretion was 753 mg. and the daily average 52 mg. Indol 250 mg. daily was then administered. The output of bilirubin rose promptly to a peak of 190 mg. daily and then stabilized at just over 100 mg., about double the output during the control period. The total output for the 14 days while indol was given was 1,701 mg. and the daily average 121 mg. The blood decreased from 4,370,000 erythrocytes and 67 per cent hemoglobin to 3,200,000 and 49 per cent hemoglobin. There was no significant change in the number of circulating reticulocytes. When the excretion of bilirubin and the blood levels had apparently stabilized, the amount of indol was increased to 500 mg. daily. This move resulted in an increase in the output of bilirubin to a total for 7 days of 1,406 mg., a daily average of 200 mg. At this point the blood had dropped to 2,700,000 erythrocytes and 39 per cent hemoglobin, again with no increase of reticulocytes. The diet was then supplemented with 10 gm. of liver extract daily, the administration of 500 mg. of indol daily being continued. Following the liver extract the reticulocytes rose on the 6th day to 9 per cent, and on the 8th day to 22 per cent followed by a progressive increase in blood levels. The output of bilirubin dropped progressively to an average of 100 mg. daily when infection intervened and the experiment was terminated. Here again the addition of liver extract reduced, by about 50 per cent, the hemolysis by indol.

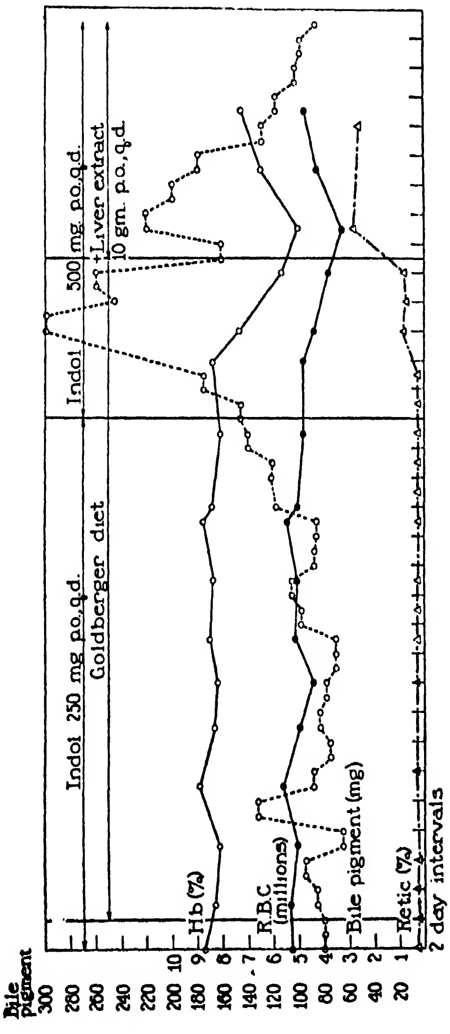
Experiment 4 (Text-Fig. 4).—In this experiment the administration of indol, 250 mg. daily, was begun while a normal diet was fed. During the control period without indol of 22 days, 1,643 mg. of bilirubin were excreted, a daily average of 74 mg. During a corresponding period of 22 days during which 250 mg. of indol were fed daily, the total output of bilirubin was 2,262 mg., a daily average of 101 mg. There was no apparent change in the blood. This animal showed a slight though distinct susceptibility to the hemolytic effect of indol even though on a normal diet. As the animal became adjusted to the presence of the toxin the



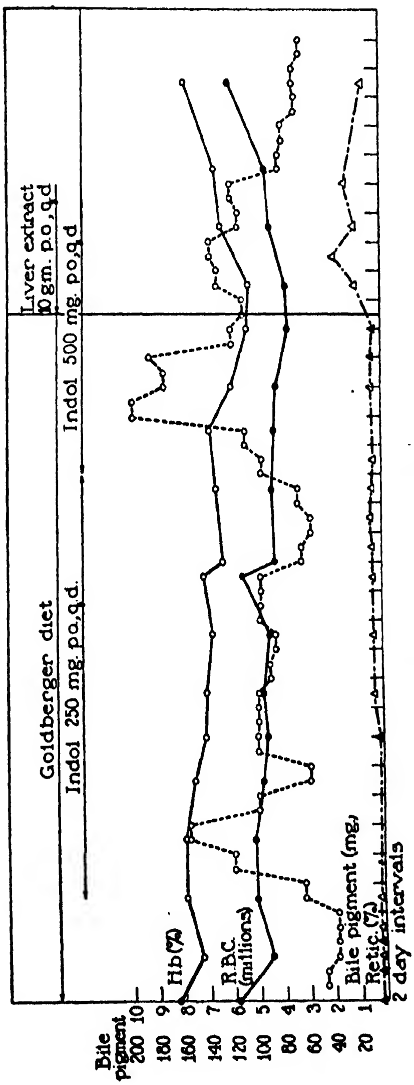
TEXT-FIG. 2. Dog 2



TEXT-FIG. 3. Dog 3



TEXT-FIG. 4. Dog 4



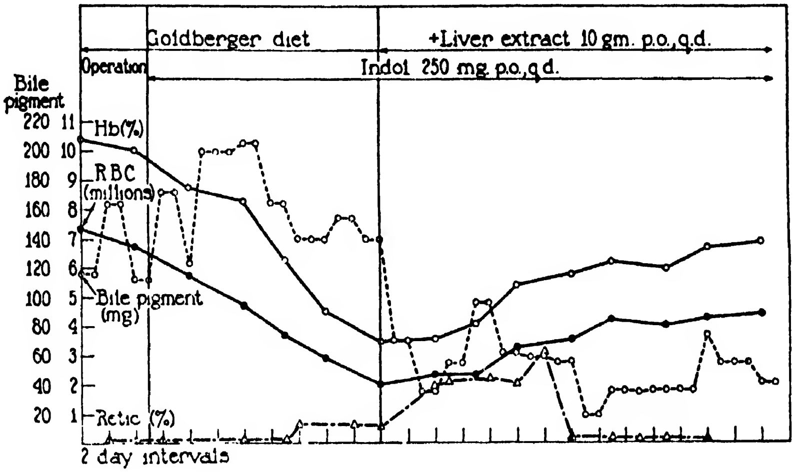
TEXT-FIG. 5. Dog 5

normal levels of the output of bilirubin were again restored, an average of 81 mg. a day for 20 days. At the beginning of Text-fig. 4, the diet was then changed to the deficient régime and the indol was continued. There ensued a slow but steady increase in the excretion of bilirubin over a period of 33 days. The total output for the first 10 days of this period was 868 mg. and for the last 10 days 1,160 mg. Coincidentally the blood dropped from 5,320,000 erythrocytes and 83 per cent hemoglobin to 4,800,000 and 81 per cent without any increase of reticulocytes. The daily dose of indol was then increased to 500 mg. The output of bilirubin for the next 10 days was 2,150 mg. and the blood dropped to 3,250,000 erythrocytes and 50 per cent hemoglobin. Without any other change 10 gm. of liver extract were administered daily. On the 2nd day the reticulocytes rose to 28 per cent followed by a rise in blood levels in 6 days to 4,200,000 erythrocytes and 65 per cent hemoglobin. The output of bilirubin dropped steadily to give a total of 1,657 mg. for the 10 days after liver extract, and to 608 mg. for the subsequent 6 days, an average of 101 mg. daily as compared to 215 daily before liver was given.

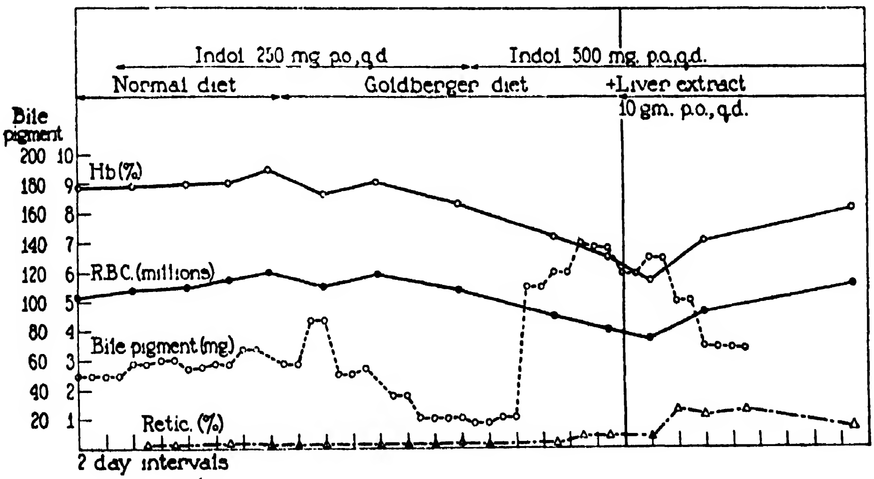
Experiment 5 (Text-Fig. 5).—This animal was fed the deficient diet for 1 month before indol was begun in a dosage of 250 mg. daily. During a control period of 12 days on this diet a total of 722 mg. of bilirubin were excreted, an average of 60 mg. daily. During the first 12 days of the indol feeding, 1,232 mg. of bilirubin were excreted, an average of 100 mg. daily. This increased hemolysis was compensated for by erythropoietic activity since no decrease of blood levels appeared, although the reticulocytes increased slightly in numbers. After 30 days the dose of indol was increased to 500 mg. daily. The output of bilirubin jumped to 1,706 mg. for the next 12 day period, an average of 140 mg. daily. The blood dropped from 4,390,000 erythrocytes and 67 per cent hemoglobin to 3,200,000 erythrocytes and 54 per cent hemoglobin without any increase in reticulocytes. Liver extract, 10 gm. daily, was then given. On the 2nd day following the reticulocytes were 9 per cent and on the 4th day 18 per cent. The output of bilirubin decreased somewhat more slowly than had been the case previously. For the first 10 days after the institution of liver therapy 1,208 mg. were excreted, a decrease of only 14 per cent, but in the next 7 days the output was 530 mg., an average of 75 mg. daily, a decrease of 50 per cent. Concurrently the blood rose in 6 days to 4,420,000 erythrocytes and 64 per cent hemoglobin and in the next 10 days to 5,400,000 erythrocytes and 75 per cent hemoglobin.

Experiment 6 (Text-Fig. 6).—This experiment is particularly instructive since the animal disliked the black tongue diet and took almost none of it after the biliary fistula was set up 1 month following the beginning of the diet feeding. Indol 250 mg. daily was started on the 6th postoperative day while the output of bilirubin was still in the 100 mg. range. The excretion of bile pigment rose sharply to a total for the next 17 days of 2,822 mg. or 166 mg. daily. The blood dropped from 6,700,000 erythrocytes and 101 per cent hemoglobin to 2,000,000 erythrocytes and 35 per cent hemoglobin. There was a slight rise of reticulocytes to 6 per cent. Liver extract, 10 gm. daily, was then administered, but the animal still refused to take the diet. The output of bilirubin dropped sharply neverthe-

less to a total of 646 mg. for the next 10 day period. On the 4th day the reticulocytes rose to 19 per cent, and on the 12th day to 30 per cent, then dropped quickly to normal levels. In the first 10 days the blood rose to 3,270,000 erythrocytes and 54 per cent hemoglobin, and in the next 10 days to 4,400,000 erythrocytes and 69 per cent hemoglobin.



TEXT-FIG. 6. Dog 6



TEXT-FIG. 7. Dog 7

Experiment 7 (*Text-Fig. 7*).—In this experiment after a primary control period of the normal diet 250 mg. indol daily were fed for 14 days. There was no increase in the excretion of bilirubin over the period without indol. The deficient diet was then fed and the small dose of indol continued. Deficiency developed so slowly however that no increase in the excretion of bilirubin was evident over a 15 day period. The indol was then increased to 500 mg. daily. During the previous

10 days the total output of bilirubin was 310 mg. During the next 10 day period the total output increased to 902 mg. or over 3 times, with a coincidental drop in blood levels from 5,490,000 erythrocytes and 82 per cent hemoglobin to 3,780,000 erythrocytes and 57 per cent hemoglobin, without any significant increase of reticulocytes. Liver extract was then given as a supplement to the diet in 10 gm. amounts daily without changing any other factor. The reticulocytes rose on the 5th day to 8 per cent and on the 7th day to 10 per cent. The output of bilirubin dropped steadily from the 4th day after liver was begun to an average of 78 mg. daily for the next 10 days. The blood rose to 4,640,000 erythrocytes and 70 per cent hemoglobin.

DISCUSSION

From the experiments the evidence is clear that an amount of indol which is well tolerated on a normal diet is causative of hemolysis and anemia when a deficient diet is fed. Furthermore the hemolytic as well as the anemia-producing effect of the combination of indol and a deficient diet may be prevented by supplementing the diet with liver extract. This is clear from the fact that following such supplement the output of bilirubin decreases to normal levels coincidently with the rise of reticulocytes and the improvement of the blood levels. It is to be inferred that the anemia results at least in part from hemolysis since that process is clearly conditioned by the dietary deficiency. The possibility exists, however, that a double process is operative, (*a*) the described increase of the hemolytic effect of indol on the deficient diet, and (*b*) a decrease in the erythropoietic power of the bone marrow. There is little evidence however that liver extract has the power to increase the rate of formation of erythrocytes since it is practically without effect in the standardized anemic dogs of Whipple (7). The anemia can be explained quite as well on the basis of simple destruction as by invoking two factors.

The mechanism of the increased hemolytic effect of indol in the presence of dietary deficiency is not clear. One possibility concerns the indol. (*a*) Under conditions of vitamin deficiency indol may be more freely absorbed than normally, (*b*) it may be metabolized to indoxyl more slowly than normally, or (*c*) an abnormal metabolism may be present by which some lytic intermediate product is formed. Experiments (8) have been made which show that no change in the rate of absorption or the conversion to indoxyl can be demonstrated even in a deficiency severe enough to cause black tongue. Moreover

it has not been possible to show any hemolytic effect of pure indol on washed erythrocytes *in vitro*. The possibility of the formation of pathological intermediate products is now under investigation.

A second possibility concerns a change in the erythrocyte itself as a result of the deficiency. It could become susceptible to lysis by indol although not normally so or it could be rendered by indol more susceptible to a normally existing hemolytic process. Both of these possibilities are now being studied.

An interesting phenomenon is the tendency of the organism to develop without treatment a slight but definite resistance to the hemolytic effect of indol. Reference to the figures shows that the increase in the rate of excretion of bile pigment is more marked immediately after the first administration of indol and then drops somewhat to a sustained effect. This factor does not affect the validity of the conclusions however since sufficiently long periods were observed to rule out any spontaneous changes.

The changes in the reticulocytes in these experiments are also of interest. It is known from the work of Steele (9) and previous workers that bleeding or the hemolysis resulting from phenylhydrazine is attended by persistently elevated levels of reticulocytes. The factor of hemolysis in the experiments with indol is indisputable but essentially no elevation of reticulocyte numbers is encountered. One possible explanation is that the hemolysis by indol involves all the hemoglobin-containing cells including the reticulocytes, whereas bleeding or phenylhydrazine removes only adult cells, leaving younger forms in the circulation. Dock (10) states that doses of saponin may be administered which cause severe anemia with low numbers of reticulocytes. Were a reticulocytolytic action of indol operative the increase in numbers of those cells with the cessation of hemolysis following liver extract would be easily explained. They are being formed actually in greater numbers than normally but are not visible because they are destroyed as fast as formed. When lysis ceases, however, they appear for a brief period until the erythropoietic activity of the bone marrow slows down following the cessation of the constant drain upon it.

The explanation is suggested then that the anemia resulting from a deficiency of the vitamin and the administration of indol is a hemo-

lytic anemia, due possibly to an increased susceptibility to lysis of all hemoglobin-containing cells, including reticulocytes. The rise in blood levels following the administration of liver extract as well as the temporary increase of reticulocytes seems to reflect decreased hemolysis and a decreased, rather than an increased production of cells. No experiment made so far, however, has ruled out conclusively the possibility that a double factor is operative, (*a*) an increased rate of cell destruction and (*b*) a lessened rate of cell production, referable either to the toxic effect of indol or to the deficient diet. Further experiments bearing on this point are in progress.

CONCLUSIONS

1. Indol is more hemolytic in the presence of a deficiency complex than when a normal diet is fed.
2. The hemolytic effect can be abolished by supplementing the deficient diet with liver extract curative of pernicious anemia in man.
3. The hemolysis affects all hemoglobin-containing cells, including reticulocytes.
4. The repair of the anemia resulting from the administration of indol in the presence of a deficiency represents the cessation of a hemolytic process.
5. An abnormally low rate of production of erythrocytes may well be a factor in the production of the anemia.

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HEPATIC DYSFUNCTION IN DOGS FED DIETS CAUSATIVE OF BLACK TONGUE

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The morphologic alterations of tissue which result from a deficiency of the thermostable fraction of the vitamin B complex have been minutely described, but little information is available concerning those disorders of function which are present during life as indicated by the postmortem appearance. Gross as well as microscopic changes of the hepatic parenchyma are frequent in canine black tongue, a condition generally assumed to be the result of a deficiency of vitamin B_2 (G), and the disease can be prevented or cured by liver extract. No evidence has been advanced, however, that the histologic changes are associated with a derangement of hepatic function, even though they may be prevented or cured by the administration of hepatic substance. Accordingly a study has been made of the ability of the liver to excrete intravenously injected bilirubin, as affected by feeding a diet lacking in vitamin B_2 (G) and causative of canine black tongue.

The rate of excretion of injected bilirubin as a test of liver function was first described by von Bergmann (1) and by his associate, Eilbott (2). The study was taken up by Harrop and Barron (3) and confirmed. The test was applied by these workers to pernicious anemia, and it was found that liver dysfunction, by the bilirubin test, was a uniform feature of that disease. Soffer (4) applied the test in a study of liver function during pregnancy, and was satisfied that it was a delicate method of detecting early hepatic insufficiency. Strauss and Castle (5) have published evidence that vitamin B_2 (G) (the factor which is preventive and curative of canine black tongue) is similar to the extrinsic antipernicious anemia factor in its distribution and resistance to heat. Hence it was desirable, in studying hepatic function during the feeding of diets causative of black tongue, to apply a test which had been shown to demonstrate hepatic dysfunction in pernicious anemia. Therefore the quantitative measurement of the ability of the liver to excrete intravenously injected bilirubin was employed, after suitable modification which allowed it to be applied to the dog.

Histologic changes in the livers of dogs dying of acute black tongue have been

described by Lillie (6). In certain animals fed a diet causative of black tongue, and with the stomatitis which is characteristic of that condition, Sebrell (7) has described a "yellow liver" associated with a very marked fatty infiltration. This pathologic change, as well as the less striking but relatively constant hepatic lesions of uncomplicated black tongue can be prevented by the addition of dried or autoclaved yeast to the diet. Goldberger (8) showed that black tongue could be prevented and cured by supplementing the diet with the liver extract, which is effective in the treatment of pernicious anemia.

Methods

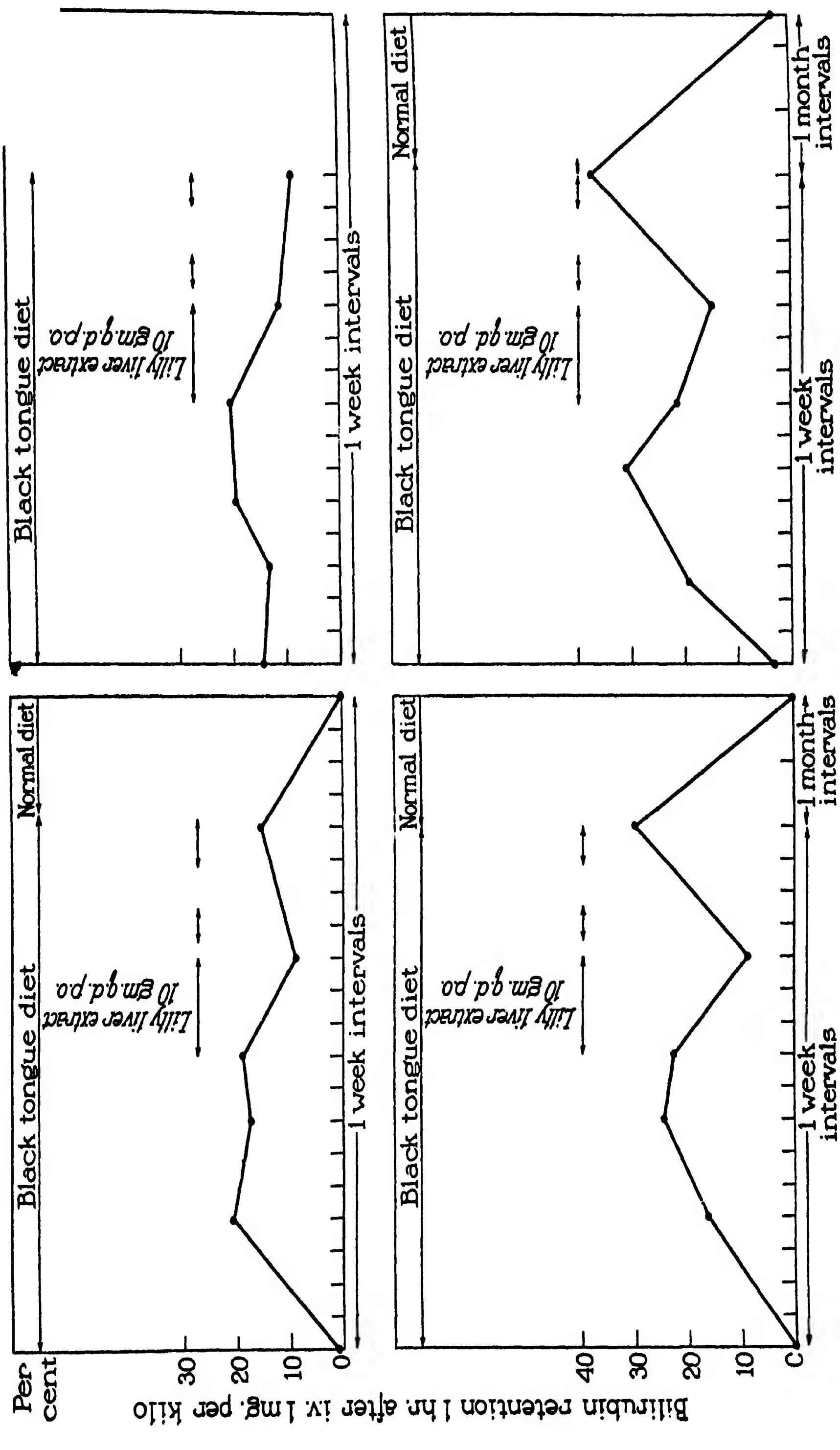
Crystalline bilirubin was obtained from the Eastman Kodak Company. Experiment showed that the dog has a much greater power to excrete injected bilirubin than does man, a rate of about 2 mg. per kilo per hour being common. In man the excretion of 1 mg. per kilo in 4 hours is considered normal.

2 mg. of bilirubin per kilo of body weight were dissolved in 15 cc. of 0.1 molar solution of sodium carbonate which previously had been brought to the boiling point and then allowed to cool to 80°C. A control sample of oxalated blood was taken; the pigment was injected and after an interval of 5 minutes, to allow thorough mixing, a second sample of blood was drawn. A third sample was taken 1 hour after injection. Perfectly dry syringes and needles were used to avoid hemolysis. The amount of bilirubin in the plasma was determined by the method of Ernst and Förster (9). The plasma was precipitated by acetone, using different concentrations according to the amount of bilirubin in the sample. After thorough shaking the mixture of acetone and plasma was centrifuged and read against the bichromate standards.[1]

The bilirubin content of the specimen taken 5 minutes after the injection, less the content of the first or control sample, was considered as containing 100 per cent of the injected pigment. The percentage of bilirubin retained in the third, or 1 hour plasma, was thus calculated after subtraction of the control figure.

The dogs were healthy mongrel animals averaging about 8 kilos in weight. The diet fed was that first described by Goldberger and used in many previous experiments (10) in this laboratory. It is known to produce acute black tongue after 5 to 12 weeks of feeding. Check tests of the ability of the animals to excrete intravenously injected bilirubin were made before the diet was fed and a normal figure was established for each animal. During the period of diet feeding the tests were repeated at intervals. The effect on the rate of excretion of bilirubin which resulted from supplementing the diet with 10 gm. of liver extract daily and from feeding the normal diet once more was tried. The liver extract was made up to a 25 per cent solution in water and was fed by stomach tube.

[1] Supplied by the La Motte Chemical Company.



TEXT-FIG. 1. The change in the rate of bilirubin excretion in dogs fed a deficient diet.

RESULTS

Four dogs were tested repeatedly to determine their power to excrete bilirubin under normal conditions, Text-fig. 1. In three there was no retention greater than 5 per cent. In the fourth animal the normal retention was between 12 and 15 per cent. In the first three instances the next test, done 4 weeks after the diet feeding was begun, showed a well defined retention ranging from 18 to 30 per cent. Test animal 4, with a normally low excretory function, showed no increase in retention at this period. At the second test done at the 5 to 7 week period of diet feeding, retention was present in all four animals and increased in three. No evidence of black tongue was present but all were losing weight.

Having established the fact of a progressive decrease in liver function during the diet feeding, the effect of supplementing the diet with 10 gm. daily of liver extract (Lilly N.N.R.) was studied. In all four animals an increased but still somewhat subnormal excretory power for bilirubin resulted.

The diet was then changed to a mixture of cooked meat, bread, and dog biscuit which is known empirically to maintain dogs in good health over a period of years. In every instance the excretory power for bilirubin was restored to normal by this move.

DISCUSSION

The results are clear cut in all four animals tested, and indicate a progressive decrease in the excretory power of the liver during the feeding of the deficient diet. Improvement in function followed the addition of liver extract, a finding in accord with the fact that liver extract is preventive and curative of canine black tongue. The incomplete restoration of normal hepatic function following the supplement with liver extract suggests that the diet is deficient in more than one factor, both of which are required for normal liver function. This is in accord with the findings of Ruffin and Smith (11) who showed that liver extract was not completely preventive of the Goldberger diet effect. The defect could be remedied in our experiments by feeding a normal mixed diet, good evidence that permanent functional damage had not been caused.

The exact nature of the lacking factor in the Goldberger diet has

not been ascertained. The recent studies of Street (12) indicate that black tongue may be caused by feeding synthetic diets lacking the heat-stable vitamin B complex other than flavin. Furthermore Koehn and Elvehjem (13) have shown that black tongue is not prevented or cured by supplementing the Goldberger diet with flavin (B_2), but that cure is effected by a substance in the B_2 complex which is not adsorbed by Fuller's earth or by alcohol and ether. They consider it similar to, if not identical with, the antidermatitis factor for the chick (filtrate factor). More accurate studies must await the identification of this substance.

CONCLUSIONS

1. The feeding to dogs of a diet lacking the vitamin B_2 (G) complex results in a lowered capacity of the liver to excrete intravenously injected bilirubin.
2. Normal function can be partly restored by supplementing the diet with liver extract.
3. Normal function can be completely restored by feeding a normal diet.

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CLINICAL OBSERVATIONS ON THE WHIPPLE LIVER FRACTION (SECONDARY ANEMIA FRACTION)

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In 1930, Whipple, Robscheit-Robbins and Walden[21] reported a liver fraction which was potent in the treatment of experimental post-hemorrhagic anemia. Their experiments were performed upon carefully standardized dogs in which a chronic anemia had been produced by repeated bleeding. During the past 3 years the effect of this fraction of liver has been studied at the hospital of this institute in 11 patients with chronic hypochromic microcytic anemia.

The fraction is the dry, insoluble material obtained from the precipitation of an acidified aqueous extract of whole liver with 70% alcohol. It represents 3% by weight of the whole liver or 10% by weight of the dried liver. The fraction was found by Whipple and associates to possess 65 to 75% of the potency of whole liver as measured by new hemoglobin production in the standard anemic dogs. They demonstrated by control experiments with iron feeding, that the iron content of the liver fraction was not wholly responsible for the hemoglobin regeneration in these dogs. This fraction of liver has been shown to be practically inert in the treatment of patients with pernicious anemia,[3] and conversely the fraction of liver soluble in 70% alcohol (Fraction G of Cohn), containing the hematopoietic principle effective in pernicious anemia, possesses only 10 to 20% of the potency of whole liver in the treatment of Whipple's anemic dogs[17] and exerts little or no therapeutic action in the secondary anemias of man.[10, 14]

Although the oral administration of whole liver and crude extracts of whole liver has proved useful in the treatment of human secondary anemias,[5, 8, 9, 11, 14, 18, 19] there have been few studies of the clinical effect of Whipple's fraction of liver (Eli Lilly and Company's, Liver Extract No. 55 with Iron); in this commercial preparation 0.5 gm. of iron and ammonium citrate (83 mg. iron as metal) has been added to

each 3 gm. of the dry powder (the amount of extract derived from 100 gm. of whole liver). In 1932, Cheney and Niemand[2] published a series of 50 cases of secondary anemia treated with the commercial preparation. Excellent results were obtained in 13 cases of post-hemorrhagic anemia with rises in reticulocyte, hemoglobin and erythrocyte levels, whereas other forms of secondary anemia were not strikingly benefited. Since the patients of this series received 230 to 700 mg. of iron as metal daily mixed in with the liver extract, it is impossible to evaluate the efficacy of the liver extract apart from its iron content in these cases, even though the authors reported that 6 of their post-hemorrhagic cases had failed to show material improvement on previous iron therapy. More recently Cheney[1] has found the same commercial preparation of value in the treatment of certain cases of anemia secondary to gastric cancer.

In the course of their study of hookworm in Puerto Rico, Rhoads, Castle, Payne and Lawson[16] treated 7 patients with the No. 55 fraction without iron. On a daily dosage of 12 gm. of the extract 4 of the patients showed reticulocyte rises of from 3.6 to 13.4% followed by moderate improvement in hemoglobin and erythrocyte levels. The 12-gm. daily dose of the liver extract was found on analysis to contain 34 mg. of iron as metal which might be sufficient to exert some hematopoietic effect and thereby account at least in part for the apparent activity of the material. In all 7 cases secondary reticulocyte responses and more rapid improvement in hemoglobin and levels of erythrocytes followed the addition of 2 gm. of ferric ammonium citrate to the daily dose of liver extract.

The lack of conclusive proof that the clinical effect of the Whipple liver fraction is not entirely due to the iron content stimulated the present clinical study. Heath[7] has stressed the individual variability in the iron requirement of patients with hypochromic microcytic anemia, pointing to one case which gave a maximal response to 85 mg. of iron a day by oral administration in contrast to 3 cases which showed no response whatsoever to a dose of 50 to 85 mg. per day given by the same route. Reimann and Fritsch[15] have reported good results with as little as 22 to 100 mg. of iron a day administered by mouth. It was in order to control the iron effect that the following method of procedure was adopted in the investigation of the clinical activity of the Whipple liver fraction.

Methods. Liver Extract No. 55, which was supplied without added iron by Eli Lilly and Company, was analyzed for its iron content by the method of Wong.[22] Several determinations revealed an iron content of 2.5 to 2.75 mg. of iron per gm. of dry powder, or 62 to 69 mg. of iron per 25-gm. dose of the extract.*

The principle of the double reticulocyte response was then employed in the study of the patients. This principle was introduced by Minot and his associates[11] to determine the optimal dosage of liver in pernicious anemia. Later Dameshek and Castle[4] used the method for assaying the relative potency of commercial liver extracts in the treatment of pernicious anemia, while Heath[7] has devised a similar comparative test for evaluating the efficacy of various iron preparations in the treatment of hypochromic anemia. As stated by Dameshek and Castle, the principle of the double reticulocyte response is briefly this: When a uniform daily suboptimal dose of a known potent material is given, the reticulocyte response is concluded or on its downward course in from 10 to 12 days. This allows for the observation of a possible second response of reticulocytes when another more potent material or a larger dose of the same material is given over a period immediately following the first period. Daily doses must be used since the stimulus to the bone marrow must be continuously applied during the estimation of the comparative potency of each product.

Applying this principle of the double reticulocyte response, we have treated patients with severe hypochromic microcytic anemia continuously over 3 consecutive periods of at least 10 days each. In the first period, each patient received about 70 mg. of iron a day as 1.7 cc. of a 25% solution of ferric ammonium citrate, in the second period 25 gm. of Liver Extract No. 55 (the amount derived from 833 gm. of whole liver) daily, and in the third period a maximal dose of inorganic iron preparation. Reticulocytes were followed throughout the first two periods in all cases, and through the third period in the majority of the cases. We have felt that after giving 70 mg. of iron a day throughout the first period we were justified in interpreting any further reticulocyte response occurring in the second period as due to some factor or factors in the liver fraction other than the contained iron. In other words, we have looked upon the first period as controlling the iron content of the liver fraction.

Case Material. Eleven cases of severe hypochromic anemia (Table 1) were studied in the manner just described: 7 patients were females; 4 males. The ages varied from 20 to 70 years. Chronic blood loss was regarded as the chief etiologic factor in the first 7 patients, 3 of whom were suffering from chronic ulcerative colitis. Gross blood loss of sufficient magnitude to stimulate a reticulocyte response was not observed in any of these 7 patients during the period of observation. Cases 8 and 9 showed gastric hypochlorhydria, associated with a gastro-enterostomy in Case 8. Case 10 presented the typical picture of nutri-

* We are indebted to Dr. Walther Goebel for the information that the iron in the liver fraction is chiefly in the ferrous state, although a small portion is ferric iron.

tional anemia resulting from an inadequate iron intake over a period of years, while Case 11 was a man with carcinoma (probably of the prostate) with multiple metastases to bone.

The original erythrocyte levels of the 11 patients varied from 2,430,000 to 4,600,000 red blood cells per c.mm., while the hemoglobin levels ranged from 30 to 56%. In 9 cases the hemoglobin level was below 50%. The mean corpuscular volume and color index were well below normal in all 11 cases, averaging

TABLE 1

Reticulocyte Peak Obtained with Small Doses of Iron, Liver Extract and Large Doses of Iron

Patient.	Sex.	Age.	Diagnosis.	Blood picture at start of test period.					Reticulocyte peak (%).		
									I.	II.	III.
				R.B.C. (in millions).	Hemoglobin (%).	M.C.V. (μ^3).	C.I.	Reticulocytes (%).	70 mg. iron q.d.	L.E. No. 55 s iron, 25 gm. q.d.	Large dose of iron q.d.
1. F. P.	M	41	Ulc. colitis	4.05	37	58	0.46	0.8	1.6	4.0	11.2
2. H. B.	M	21	Ulc. colitis	3.18	46	73	0.72	1.4	6.0	12.5	
3. A. Z.	F	27	Ulc. colitis	2.61	30	69	0.58	2.0	7.0	6.8	12.1
4. E. T.	M	20	Banti's disease Hematemesis	4.60	56	61	0.61	1.8	4.2	5.6	
5. O. L.	F	45	Ca. of small intestine	2.43	41	78	0.84	0.6	4.0	9.8	9.0
6. E. W.	F	58	Ca. of cecum Achlorhydria	4.14	45	59	0.54	1.8	4.5	3.9	5.7
7. M. M.	F	29	Myomata uteri	3.82	51	68	0.67	0.4	2.4	15.8	8.0
8. M. B.	F	47	Gastro-enterostomy Hypochlorhydria	3.40	40	65	0.58	0.4	1.2	3.0	
9. C. L.	F	55	Hypochlorhydria	2.74	34	62	0.62	1.8	4.8	5.2	
10. H. P.	F	70	Nutritional anemia	3.31	43	70	0.65	1.2	5.4	5.2	3.0
11. L. B.	M	64	? Ca. of prostate; metastasis to bone	3.00	39	68	0.65	0.2	3.0	4.2	4.4
Average				3.39	42	66	0.63	1.2	4.0	6.9	7.6

66 μ^3 and 0.63 respectively. The reticulocyte levels at the start of the observation period varied from 0.2 to 2%. Thus these 11 patients all satisfied the usual criteria of hypochromic microcytic anemia with reticulocytes stabilized at a low level when the therapeutic tests were started.

Results. Eight of the 11 patients showed at least a slight reticulocyte response to the oral administration of 70 mg. of iron a day during

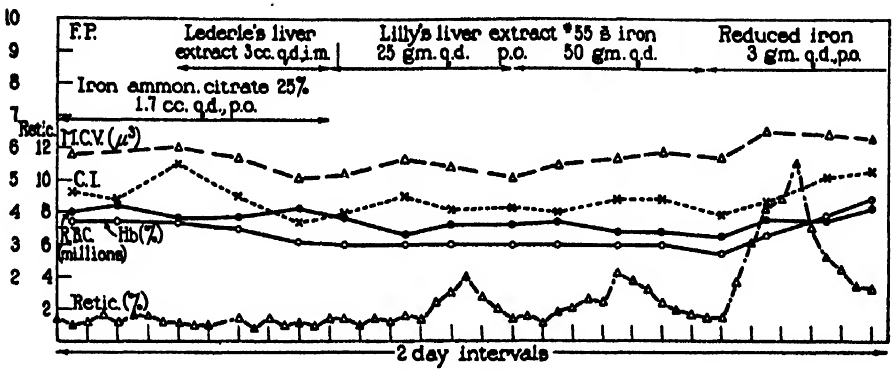

FIG. 1.—Case 1. F. P., male, aged 41. Chronic ulcerative colitis. No reticulocyte response to 70 mg. of iron a day or to antipernicious anemia liver fraction. Slight reticulocyte response to "secondary anemia" liver fraction and excellent response to large doses of reduced iron.

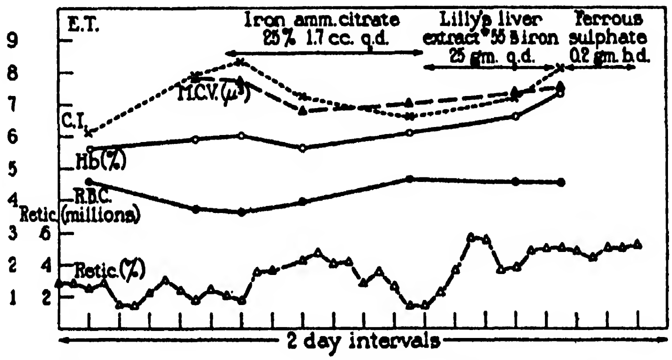

FIG. 2.—Case 4. E. T., male, aged 20. Banti's disease. Large hemorrhage 3 weeks before admission. Reticulocyte rise to 4.2% during control period; secondary rise to 5.6% during liver extract period.

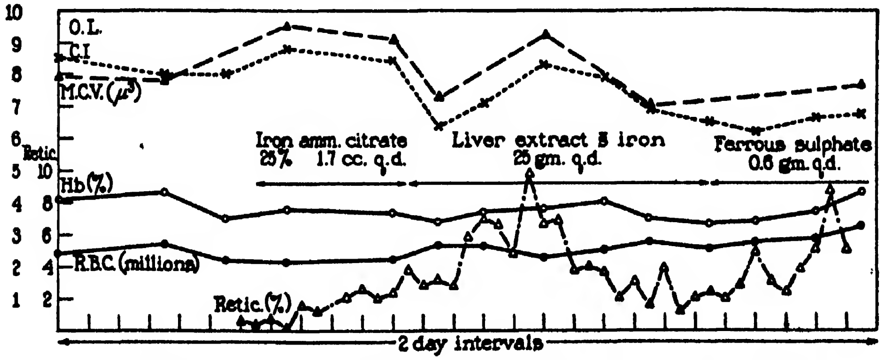

FIG. 3.—Case 5. O. L., female, aged 45. Carcinoma of small intestine. Reticulocyte rise to 4% during iron control period; further rise to 9.8% during liver extract period; tertiary response of 9% to 0.6 gm. of ferrous sulphate daily.

the control period. These responses varied from 1.2 to 7% and occurred between the fourth and tenth day after the initial dose of iron. Following the peak, the reticulocyte curve either declined or

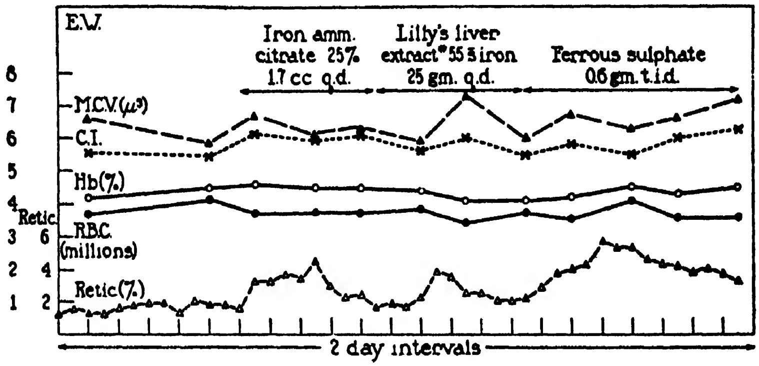

FIG. 4.—Case 6. E. W., female, aged 58. Carcinoma of cecum, histamine achlorhydria. Slight reticulocyte rises in each of the three consecutive observation periods without appreciable improvement in erythrocyte or hemoglobin levels.

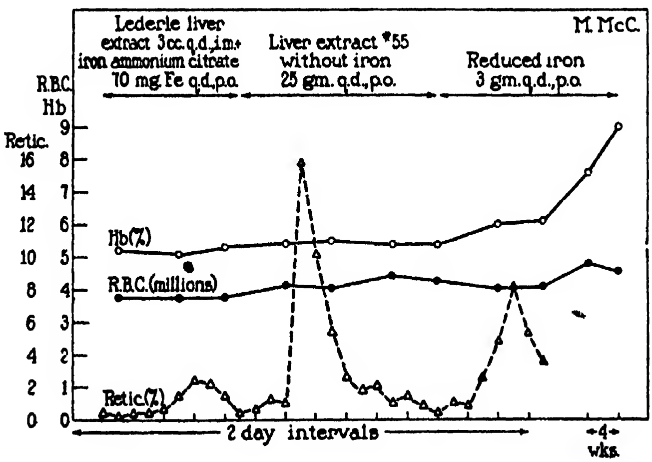

FIG. 5.—Case 7. M. McC., female, aged 29. Myomata uteri with previous hemorrhage. No blood loss during test period. Slight reticulocyte response to 70 mg. of iron a day; sharp secondary reticulocyte rise to 15.8% on the liver extract; moderate tertiary response to reduced iron in large dosage followed by rapid rise in hemoglobin.

flattened out until 25 gm. of Liver Extract No. 55 daily were substituted for the inorganic iron preparation in the second observation period. Then each of the 11 cases gave a secondary reticulocyte response to this liver fraction, the peak of the rise ranging from 3 to

15.8%. A moderate rise in levels of erythrocytes and hemoglobin occurred during these first two periods of therapy; however, the rate of improvement in blood levels was much more rapid in the third

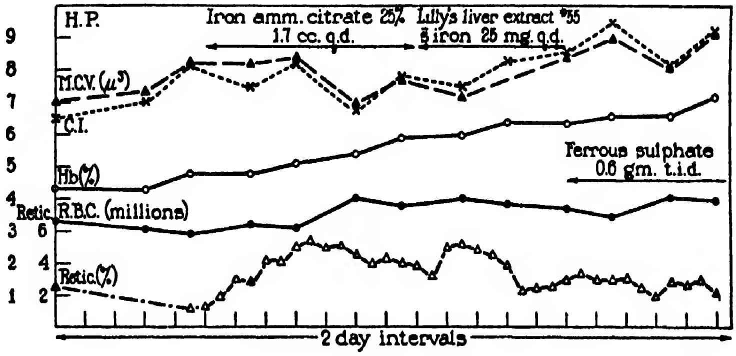

FIG. 6.—Case 10. H. P., female, aged 70. Nutritional anemia. Reticulocyte response of 5.4% to 70 mg. of iron and secondary response of 5.2% to the liver fraction. Steady rise in hemoglobin level.

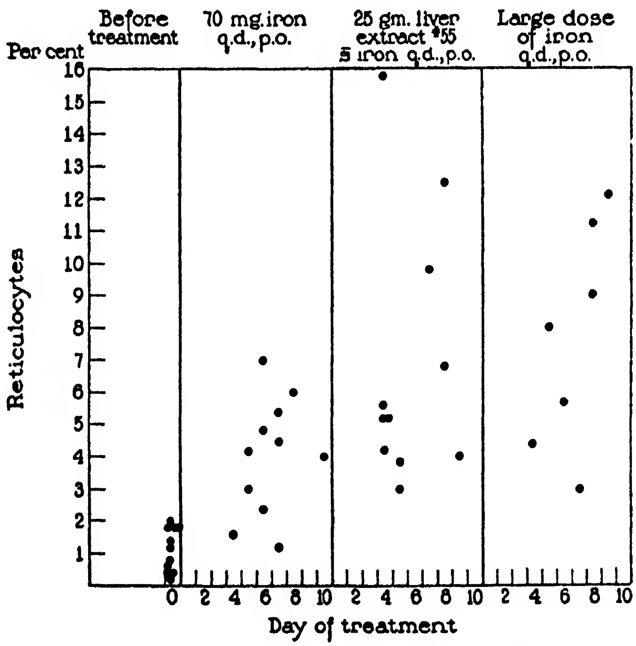

FIG. 7.—Composite chart of 11 cases. Reticulocyte peaks plotted against days on which these peaks occurred during the three consecutive observation periods.

period when the patients were receiving large doses of inorganic iron. Of the 7 cases where reticulocytes were followed throughout the third

period 5 developed tertiary reticulocyte rises which equalled or excelled the primary and secondary responses. Examples of the blood changes taking place during the three consecutive test periods are presented in Figures 1 to 6, while Figure 7 and Table 1 summarize the data for the entire series of 11 cases.

Discussion. It is quite apparent from examination of Table 1 and the text figures that all 11 cases of hypochromic microcytic anemia showed at least slight secondary reticulocyte responses to the No. 55 liver extract, which averaged 2.9% higher than the original response to the 70-mg. dose of iron. These figures suggest that the secondary anemia liver extract contains clinically active hematopoietic material apart from its iron content. The nature of this material is not known. Patek and Minot[13] have described reticulocyte responses to the oral administration of bile pigment following a period of iron medication in cases of hypochromic microcytic anemia, and Patek[12] has reported similar reactions to the administration of chlorophyll products following a suboptimal dosage of iron. These observations have been interpreted as evidence that the human body may utilize the pyrrols present in bile pigment and in chlorophyll for the formation of new hemoglobin. As early as 1925 Whipple and Robscheit-Robbins[20] concluded that parent pigment substances are stored in the normal liver. It may be that the No. 55 liver fraction supplies such parent pigment substances for new hemoglobin formation. On the other hand, it is conceivable that this liver fraction may improve the absorption or utilization of iron.

In view of the fact that the iron contained in the liver fraction is chiefly ferrous iron whereas ferric iron was administered in the control period, it is possible that the secondary reticulocyte rise may be attributed to the recognized superiority of ferrous over ferric iron in the treatment of hyperchromic anemia. How much of the iron contained in the liver fraction is available and absorbed is not known.

Hart *et al.*[6] have shown that the effect of whole liver or liver extracts on nutritional anemia of the rat produced by a diet of milk is directly proportional to the available content of iron and copper. The iron-copper ratio of the liver fraction used in our experiments is not known. It is possible that the iron-copper ratio was brought more nearly to normal by the administration of the liver fraction;

this may be the explanation of the reticulocyte rise. Our present studies furnish no data to substantiate or disprove any one of these hypotheses as to the mechanism of action of the liver fraction.

The tertiary reticulocyte responses to maximal doses of inorganic iron and the more rapid improvement in blood levels on this form of treatment may be regarded as further evidence that iron in adequate amounts is the most effective therapeutic agent in the hypochromic microcytic anemias of man.

Summary and Conclusions. The secondary anemia liver fraction of Whipple and his coworkers has been administered orally to 11 patients with chronic hypochromic microcytic anemia. A rise in circulating reticulocytes occurred in every case.

Through the application of the principle of the double reticulocyte response to control the iron content of the liver fraction, it appears likely that the liver fraction contains reticulocytogenic material apart from its iron content.

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THE EXCRETION OF PORPHYRIN IN REFRACTORY AND APLASTIC ANEMIA

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Technical procedures which have been developed for quantitative measurements of the rate of excretion of the porphyrins have advanced the knowledge of the fundamental nature of certain anemic states. By such studies the disorders of hematopoiesis may be divided into two major groups on a pathological-physiological basis. In one group the metabolism of the pigments is quantitatively increased but is normal in type. Pernicious anemia, congenital hemolytic jaundice, and the anemia caused by phenylhydrazine are all of this variety (1, 2, 3, 4, 5). In a second group, however, a qualitative as well as a quantitative deviation from the normal metabolism of pigments exists. The anemia of lead (6, 7, 8) and salvarsan poisoning (9), and of certain hepatic cirrhoses, notably hemachromatosis, are of this type (1, 10).

Recent advances in the treatment of pernicious anemia and the anemias of a deficiency of iron and copper have made prominent a group of blood dyscrasias which does not respond to the administration of liver extract or of iron. Cases of this type which are apparently primary are termed aplastic, aregenerative or achrestic anemia, and compose a group of general as well as of physiological interest.

The cause of primary aplastic anemia is obscure in most instances, although certain cases are encountered in which exposure to benzol, gold, or arsphenamine seems to have been etiological. Histological study of the hematopoietic bone marrow in these toxic cases shows no consistent difference from the lesions in patients clinically similar but who give no history of exposure to potentially toxic compounds.

Aplastic anemia is, then, therapeutically unlike pernicious anemia or the microcytic anemias, and certain cases seem to be toxic in origin. Studies of the metabolism of the pigments in cases of aplastic anemia

should establish conclusively whether the underlying physiological disturbance is like that of the deficiency or of the toxic group.

In the course of a study of refractory anemia, detailed observations of the metabolism of pigments have been made in six patients. Studies of one such case have been reported by Brugsch (11). The cases were selected to represent as closely as possible the different subgroups into which aplastic anemia may be divided (12).

To render the rationale of this study and the validity of the results intelligible, the brief review by Dobriner and Rhoads of the chemistry of the porphyrins should be consulted (13).

METHODS

The chemical and physical procedures which have been employed for the qualitative and the quantitative determinations of the excretion of porphyrins are those which have been reported previously (1, 2). The total outputs of urine and stools have been collected with toluol as a preservative for periods of three days and aliquot fractions analyzed. Determinations of the content of urobilin have been made by the method of Watson (14). The excretion of porphyrin is expressed in micrograms for the daily average of each 3-day period, the excretion of urobilin in milligrams. In the figures, the average levels of daily excretion are presented for the various periods of treatment.

Case 1 (*H. D.*) (*Figure 1*). A 66-year old female with anemia of 3 years' duration. The past history was irrelevant except for a thyroidectomy for hyperthyroidism 8 years previously. Blood transfusions had been given at intervals of 30 days and liver extract administered orally and parenterally without effect. Basal metabolic rate was −6. Throughout the period of observation the erythrocytes varied from 900,000 to 1,200,000, the hemoglobin from 15 to 23 per cent (100 grams = O_2 combining capacity 19.6 volumes per cent), and the leukocytes from 1,600 to 3,400. The differential leukocyte count was not remarkable. The reticulocytes were always less than 1 per cent. The platelets numbered 290,000, and the resistance of the erythrocytes to hypotonic salt solution was normal. A biopsy of the sternal bone marrow showed practically complete aplasia of the hematopoietic tissue, only scattered groups of primitive cells remaining.

Quantitative determinations of the excretion of porphyrin were carried out for a period of 36 days, terminating 2 weeks before death. During a 9-day control period the total coproporphyrin output was 210 micrograms per day, and of this 89 micrograms were in the urine and the remainder in the feces. These levels are appreciably lower than are the normal levels of excretion in adults. The patient

was then transfused and the average output of coproporphyrin for the second 9-day period rose to 368 micrograms, of which 114 were in the urine. This increase may possibly be due to the transfusion, for in the subsequent two 9-day periods the total output of coproporphyrin averaged 253 and 182 micrograms per day respectively. One determination of urobilin averaged 83 mgm. per day for a 3-day period, a level distinctly lower than normal.

The excreted coproporphyrin was identified by the determinations of melting point as coproporphyrin I (235°C.). The presence of Type III coproporphyrin was suggested by the chemical reactions but could not be proved by determinations of

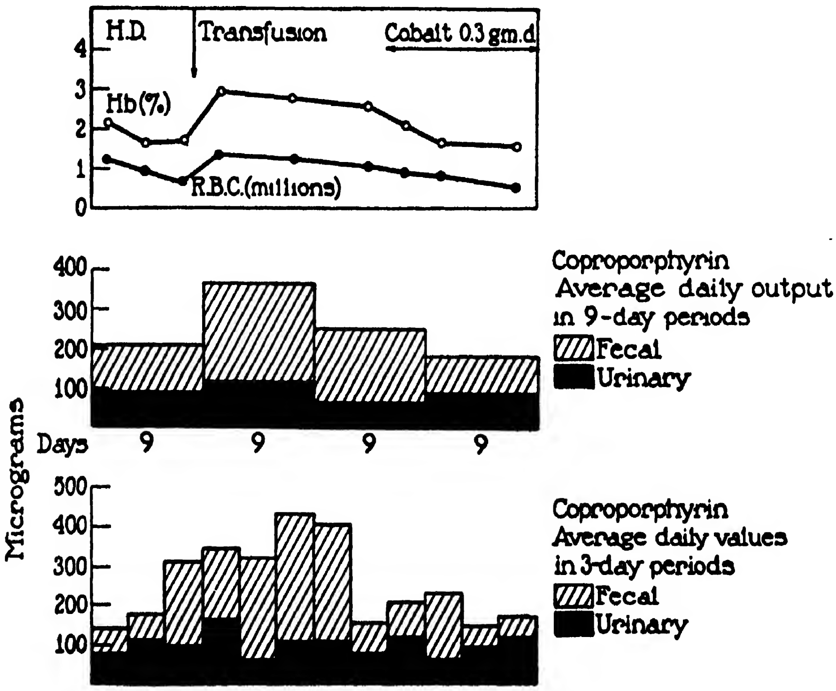

FIG. 1. Coproporphyrin Excretion in Case 1

the melting point because of lack of material. Besides coproporphyrin, the stools contained small amounts of protoporphyrin and deuteroporphyrin.

Case 2 (*C. S.*)[1] (*Figure 2*). A 54-year old teacher with anemia of 3 years' duration. The past history revealed that she had dyed her hair regularly for 15 years with a paraphenylendiamine compound. Over a period of seven and one-half months she had received eleven blood transfusions and various forms of liver extract

[1] The patient C. S. was observed both at The Strong Memorial Hospital in Rochester, New York, and at The Hospital of The Rockefeller Institute, and the authors wish to express their appreciation of the assistance of Dr. W. S. McCann in this matter.

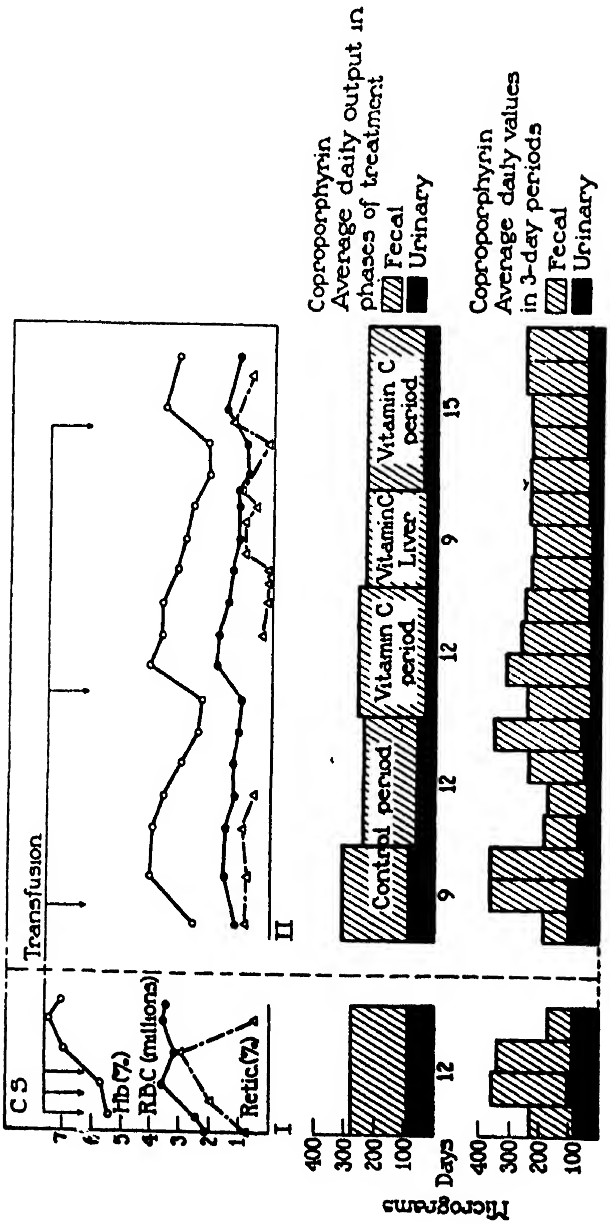

FIG. 2. Coproporphyrin Excretion in Case 2

without effect. On admission the red blood cells numbered 1,230,000, the mean corpuscular volume 100, the hemoglobin was 35 per cent, leukocytes 800, the reticulocytes 0.9 per cent, platelets 38,000 per mm.^{3}, and the fragility of the red blood cells was normal. Free hydrochloric acid was present in the gastric juice, and tests of the ability of the liver to excrete bilirubin and to convert sodium benzoate to hippuric acid showed no abnormality.

A biopsy of the sternal bone marrow revealed a moderately cellular marrow with a very marked lack of maturation of the hematopoietic cells. Practically no cells of a more adult type than primitive, endothelial or blast forms were present.

During the first period of observation, the patient was transfused three times and received various forms of treatment. The total output of coproporphyrin averaged 278 micrograms per day; of this, 92 micrograms were in the urine. Small amounts of protoporphyrin and deuteroporphyrin were present in the feces. The output of coproporphyrin increased somewhat after the transfusions.

The second period of observation was of 54 days. During the first 9-day control period the total output of coproporphyrin averaged 310 micrograms per day, of which 87 were in the urine. During this time the patient was transfused, and the excretion of coproporphyrin increased. Because of this fact a second control period of 12 days was used, during which the coproporphyrin averaged 243 micrograms per day, of which 65 were in the urine. The latter value is appreciably lower than normal. During the remainder of the study the patient was transfused several times, treated with vitamin C and with liver extract, without significant change in the excretion of coproporphyrin. The urobilin averaged 133 mgm. per day for a 3-day period.

The excreted porphyrin was identified by the melting point as coproporphyrin I (222°C.) and small amounts of coproporphyrin III (143°C.).

Case 3 (*F. Y.*) (*Figure 3*). A 30-year old housewife with anemia of 10 years' duration. When 12 years of age she developed a brown mottling of the skin of the neck and upper chest. A severe toxemia of pregnancy ending with a miscarriage occurred at 20. At this time the hemoglobin was 27 per cent and transfusions were given followed by reactions. The hemoglobin rose gradually to 65 per cent, but chronic anemia persisted for several years until the occurrence of a second pregnancy 18 months before admission, when there was an exacerbation and the hemoglobin fell to 33 per cent. The pregnancy was terminated and the patient improved. Another pregnancy one year before admission followed a similar course. On admission the erythrocytes numbered 2,400,000, the hemoglobin was 70 per cent, the mean corpuscular volume 116, the white blood cells 2,250, the reticulocytes 2.5 per cent and the platelets 55,000. The icteric index was 5, and the fragility of the erythrocytes was normal. Tests of liver function were normal. The liver and spleen were not enlarged.

A biopsy of the sternal bone marrow was made and histological examination of the tissue revealed a moderately cellular structure with a well defined predominance of young cell forms. No suggestion of leukemia was seen. The hematopoietic islands were somewhat larger than normal and a diffuse infiltration of immature cells was present.

During a 9-day control period the output of coproporphyrin averaged 417 micrograms per day, of which 220 were in the urine. In the second 9-day period the patient was treated with 1 gram of vitamin C daily, and the total coproporphyrin fell to 277 with a marked drop in the porphyrins of the urine to 68. The excretion in the feces did not change. In the subsequent 9-day periods, the first untreated, and the second a vitamin C period, the total excretion of coproporphyrin did not change appreciably, although the porphyrin in the urine rose to 100 and 110 micrograms respectively. During the next 12 days of observation the patient received vitamin C, raw liver, and ventriculin. The average daily output of coproporphyrin

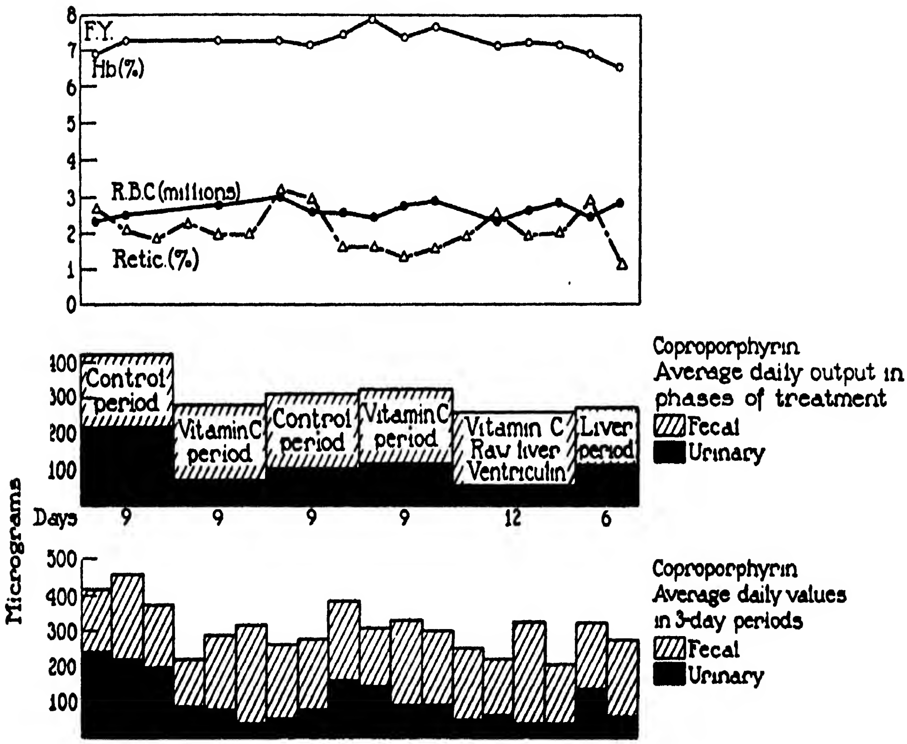

FIG. 3. Coproporphyrin Excretion in Case 3

decreased to 250 micrograms, of which 47 were in the urine. During the final 6 days of observation the patient received liver extract intravenously, and the total average daily output of coproporphyrin was 262 micrograms as compared to 250 in the previous period, but the porphyrin in the urine increased somewhat.

The excretion of urobilin averaged 99 mgm. daily.

Small amounts of protoporphyrin were present in the feces. The coproporphyrin excreted was identified as coproporphyrin I, melting point 225°C., and coproporphyrin III by a melting point of 127°C.

Case 4 (*G. M.*) (*Figure 4*). A 34-year old ship steward was admitted with anemia of one year's duration. He had had malaria in 1917 and 9 years prior to admission

he developed psoriasis, which was treated by x-ray therapy from 1930 to 1935. One year after the last treatment he developed fever and weakness and was admitted

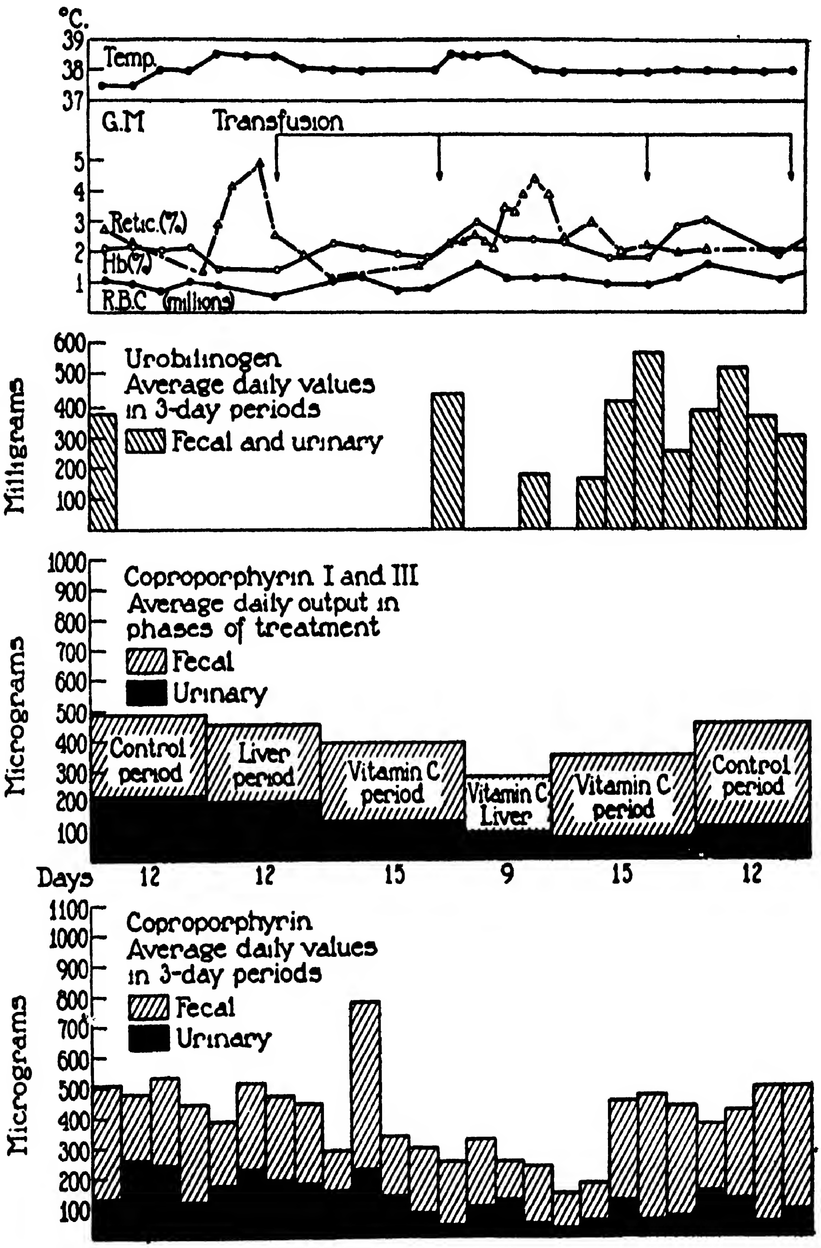

FIG. 4. Coproporphyrin and Urobilinogen Excretion in Case 4

to another hospital with a severe anemia which was refractory to treatment. Physical examination on admission to this institution was not remarkable. The erythro-

cytes numbered 1,640,000, the hemoglobin was 30 per cent, the mean corpuscular volume 84, the platelets 136,000, and the reticulocytes 5 per cent. Free hydrochloric acid was present in the gastric juice, tests of liver function were normal, the icteric index was 6. The fragility of the erythrocytes was normal. The patient had received eleven transfusions.

A biopsy of the sternal bone marrow revealed a normal degree of cellularity, and active hematopoiesis was apparently proceeding. No marked interference with maturation of hematopoietic cells was apparent.

Quantitative determinations of the excretion of coproporphyrin were made for a period of 75 days. Besides coproporphyrin relatively large amounts of protoporphyrin and small amounts of deuteroporphyrin were present in the feces.

During a 12-day control period the total excretion of coproporphyrin averaged 492 micrograms, and of this 212 were in the urine, both definitely elevated values. The urobilin excretion averaged 380 mgm. per day, a high level. During the subsequent 12-day period liver extract was given intramuscularly without significant effect on the excretion of porphyrin. For the next 15 days the patient received 1 gram of ascorbic acid by mouth daily. The excretion of coproporphyrin in the urine decreased to an average of 126 micrograms per day, and the total output of coproporphyrin to 395 micrograms. The output of coproporphyrin in the feces remained constant, and the observed decrease in the total output was consequent to the decreased amount in the urine. Except for one high value, the levels during this period were lower than in the control and in the previous liver period. In a subsequent 9-day period concentrated liver extract and 1 gram of vitamin C were administered daily. The average output for this period decreased to 275 micrograms, of which 93 were in the urine, levels which are distinctly lower than normal. This period was followed by a 15-day period during which 1 gram of vitamin C alone was administered daily. The total output of coproporphyrin rose to average 343 micrograms per day. The rise was principally in the porphyrin of the feces, the urinary coproporphyrin averaging 68 micrograms. The final 12 days of observation were utilized as an untreated control period and the total excretion of coproporphyrin increased again to an average of 455 micrograms per day, approaching the values of the first control period.

During the period of study the patient was transfused 4 times without significant change in the rate of excretion of coproporphyrin.

The levels of the excretion of urobilin following liver extract and vitamin C decreased, and following the termination of therapy, increased to the level before treatment.

The excreted coproporphyrin was proved to be a mixture of coproporphyrin I (233°C.) and III (138/165°C.) by melting point determinations.

Case 5 (*W. G.*) (*Figure 5*). A 26-year old male was admitted with severe anemia of 6 years' duration. There was a bilateral orchidectomy in 1924 for one undescended and one strangulated testicle, and in 1931 a severe refractory anemia developed. Since November 1935 the patient had received repeated transfusions. On admission there was brown pigmentation of the skin, particularly on the exposed areas. The liver and spleen were enlarged.

A biopsy of sternal bone marrow, when studied histologically, revealed an active, cellular tissue crowded with hematopoietic cells in all stages of development. Both the erythropoietic and the myelopoietic series were represented in the hyperplasia. No suggestion of leukemia was seen.

The erythrocytes numbered 2,010,000, and the hemoglobin 40 per cent. The leukocytes were 2,400, the platelets 250,000, and the reticulocytes 0.8 per cent.

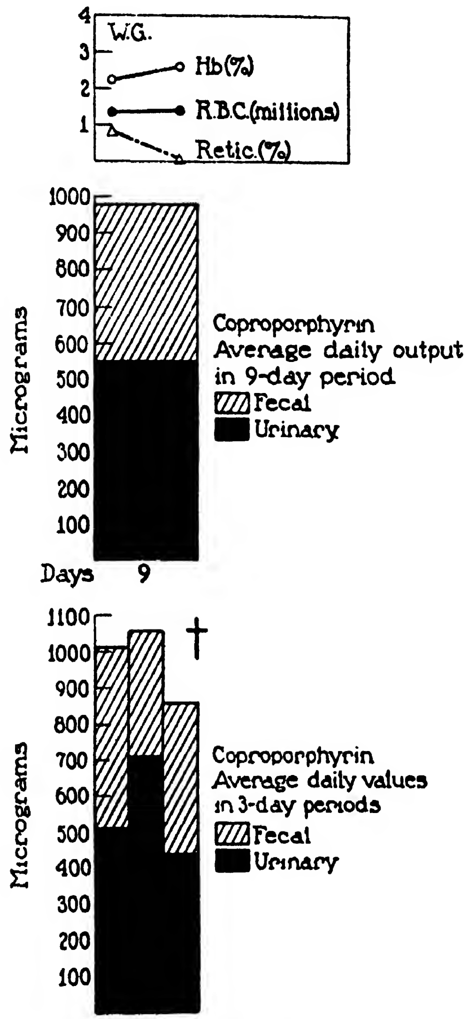

FIG. 5. Coproporphyrin Excretion in Case 5

The fragility of the erythrocytes was normal. In spite of blood transfusions the patient became rapidly worse, the erythrocytes decreasing to 1,420,000 and the hemoglobin to 27 per cent. The leukocytes fell to 1,300, and no reticulocytes were demonstrable.

The output of coproporphyrin averaged 978 micrograms per day, and of this

550 were in the urine. These levels are approximately three times normal. The urobilin averaged 273 per day for a period of 3 days.

The excreted porphyrin was proved to be a mixture of coproporphyrin I (232°C.) and III (140°C.) by determinations of the melting points.

Case 6 (*A. C.*) (*Figure 6*). A single male photographic worker of 22 with anemia of 2 months' duration. In his work this patient was exposed to hydro-

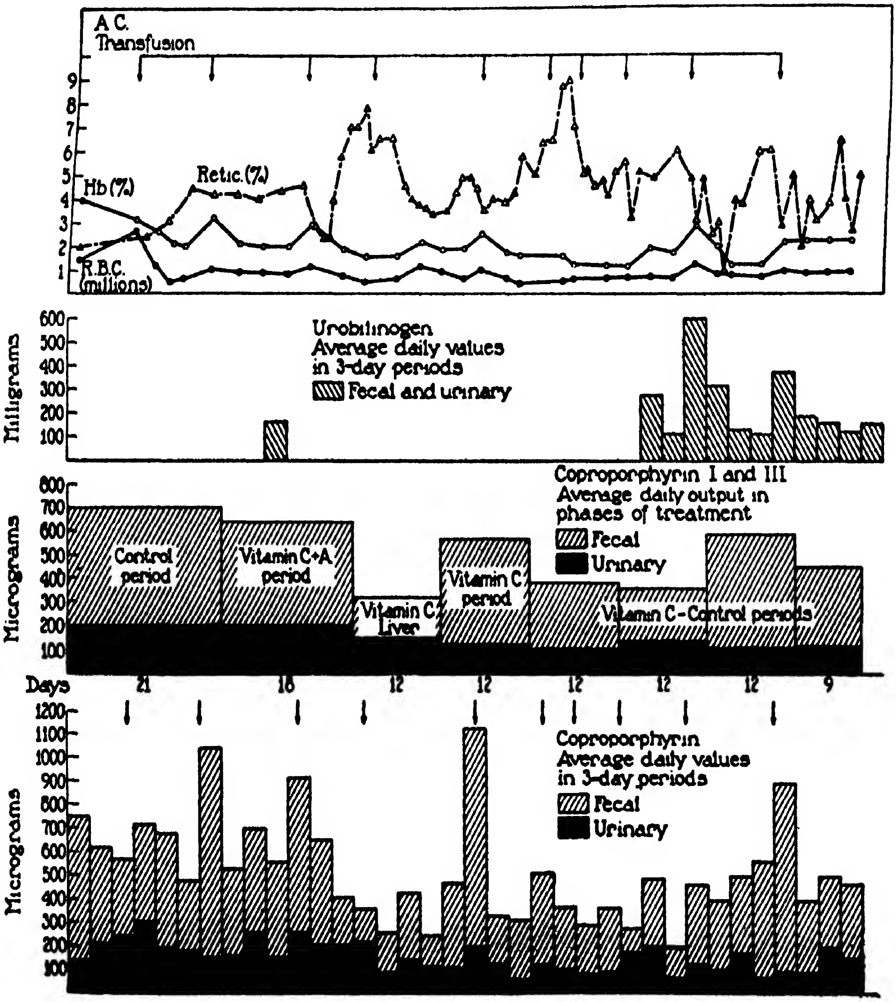

FIG. 6. Coproporphyrin and Urobilinogen Excretion in Case 6

quinone and other potentially toxic agents. Onset with epistaxis, weakness, purpura, and pallor. Six transfusions were given before admission, also liver extract intramuscularly and iron without effect. Erythrocytes 1,200,000, hemoglobin 25 per cent, leukocytes 4,150, mean corpuscular volume 116, reticulocytes 4 per cent, platelets 166,000 per mm.[3] Fragility of the erythrocytes was normal.

Free hydrochloric acid was present in the gastric juice and tests of liver function gave normal values.

Biopsy of the sternal bone marrow showed practically complete aplasia of the hematopoietic tissue.

During a control period of 21 days the patient excreted daily an average of 694 micrograms of coproporphyrin, of which 201 were contained in the urine. In the subsequent 18 days he received 2 grams of crystalline vitamin C daily by mouth, but no effect on the excretion of porphyrin was detectable since the daily average was 627 micrograms, of which 208 were in the urine. It is striking that in the periods of 3 days each, which followed 3 transfusions of blood with febrile reaction, there was a distinct increase in the output of coproporphyrin. During the next 12 days 5 cc. of concentrated liver extract (Lederle and Co.) was administered each day intramuscularly. This was accompanied by a sharp decrease in the excretion of coproporphyrin to a daily average of 320 micrograms, of which 141 were in the urine. In 5 subsequent control periods of 12 days each with vitamin C the excretion of coproporphyrin was increased but was still definitely less than in the original control period. During this time the increased output of coproporphyrin following transfusion is once more apparent.

An increased amount of protoporphyrin was present in the stools and also a small amount of deuteroporphyrin.

The excreted coproporphyrin was identified as a mixture of coproporphyrin I (232°C.) and coproporphyrin III (142/165°C.) by determination of the melting point.

DISCUSSION

The quantitative and qualitative excretions of coproporphyrin are summarized in Table I, together with the levels of the excretion of urobilin, and the hematological and bone marrow findings.

Four cases (Numbers 2, 4, 5, and 6) showed a mixed excretion of Type I and Type III coproporphyrin. In 2 cases (Numbers 1 and 3), although coproporphyrin III was not definitely identified, its presence was indicated. Since only inaccurate quantitative methods for the separation of the isomers are available, the quantitative relations between coproporphyrin I and III cannot be determined. Approximately estimated, about one-fifth of the excreted coproporphyrin in Cases 4, 5, and 6 was coproporphyrin III. In Cases 2 and 3 probably less than one-tenth of the total excretion was coproporphyrin III.

The simultaneous excretion of Types I and III coproporphyrin in aplastic anemia indicates that the metabolism of pigment in that condition is different from any which has been observed in normal individuals, in hemolytic jaundice, or in pernicious anemia. Further-

TABLE I

The Rate of Excretion of Coproporphyrin and Urobilinogen, the Blood Levels, and the Type of the Bone Marrow in the Control Period As Well As the Type of Coproporphyrin Excreted

Case number	Control period	Average daily coproporphyrin excretion			Average daily urobilinogen excretion	Red blood cells	Hemoglobin	White blood cells	Bone marrow	Type of coproporphyrin excreted
		Urine	Feces	Total						
	days	*micrograms*	*micrograms*	*micrograms*	*mgm.*	*millions*	*per cent*			
1. H. D.	9	89	122	210	83	1.100	21	1000	Hypoplastic	I (and III ?)
2. C. S.	12	65	178	243	133	1.230	35	800	Hypoplastic	I and III
3. F. Y.	9	220	197	417	99	2.400	70	2250	Immature	I (and III ?)
4. G. M.	12	212	280	492	370–450	1.110	22	950	Normally cellular	I and III
5. W. G.	9	550	428	978	273	1.42	27	1300	Hyperplastic	I and III
6. A. C.	21	201	493	694	128–165	1.440	39	2500	Hypoplastic	I and III
Normals	9	87–123	205–274	306–376	150					I

more, the disturbance in aplastic anemia differs from that observed in congenital porphyria where mass excretion of Type I coproporphyrin reverses the normal ratio between the construction rate of Type I and Type III porphyrins. The mixed excretion does, however, bear some similarity to that which has been reported in certain diseases of the liver, such as pigment cirrhosis and melanosarcoma, in lead and salvarsan intoxication, and in the conditions classified as acute and chronic porphyria.

The excretion of Type III coproporphyrin indicates a faulty metabolism of pigment, and results from either faulty construction or destruction of the respiratory pigments in any stage of their genesis or breakdown. Whether either or both of these theoretical possibilities is responsible has not yet been determined.

Of the six cases studied, three showed a hyperplastic and three a hypoplastic bone marrow. The three patients with hyperplastic marrow showed an increased excretion of coproporphyrin. Of the cases with hypoplastic marrow two showed a decreased excretion, and one a marked increase. No explanation of the last case is at hand. In only one case was an apparently definite effect of vitamin C on the excretion of porphyrin noted. In this instance the decreased excretion of porphyrin was due to a decrease in the coproporphyrin of the urine alone. In Cases 4 and 6 the excretion of coproporphyrin decreased following liver therapy, and was further decreased in Case 4 when liver and vitamin C were used together, although vitamin C alone produced little or no effect. The mechanism of these possible changes is not known. However, unpublished studies of congenital porphyria have shown that both liver (15) and vitamin C (16) have some effect on disturbance of pigment metabolism in this disease.

SUMMARY AND CONCLUSIONS

1. In 4 of 6 cases of aplastic anemia studied, a mixture of Type I and Type III coproporphyrins was excreted as ascertained by determination of the melting points of the crystalline pigments. In 2 other cases the presence of the Type III compound was indicated but not proven because of lack of material.

2. The pathological excretion of Type III porphyrins suggests that aplastic anemia results from an intoxication.

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REFRACTORY ANEMIA

ANALYSIS OF ONE HUNDRED CASES

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The recent development of a clearer understanding of pernicious anemia and of the conditions which are allied to it has made more prominent the anemic states which do not respond to adequate therapy with liver extract or with iron. These refractory anemias have been long recognized but have been classified loosely as severe secondary, aregenerative or aplastic anemia. Insufficient information has been available, however, to warrant any conclusions concerning the fundamental nature of the conditions. For this reason, a clinical and experimental study of refractory anemia has been made at the Hospital of The Rockefeller Institute. In the series, all those patients have been included who were referred to the institution with anemia of severe degree which did not respond to the usual therapeutic methods. In this way a suitable cross section of the problem as it presents itself to the practitioner was obtained.

The diagnosis of refractory anemia can be made oniy rarely on hematologic evidence alone. Anemia is moderate to most severe, slight macrocytosis is a feature and the shape of the red cells may deviate only slightly from the normal or may show the most profound alterations. Leukopenia is almost always present and may be mild or extreme; it is usually associated with granulopenia and with the presence of abnormal, so-called toxic polymorphonuclear cells. Thrombopenia is usually marked but may be absent. Refractory anemia may frequently be differentiated from other anemic states by the prominence of hemorrhagic phenomena and by a tendency to the

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development of necrotic lesions of the mucous membranes. Fever is frequent and may be a prominent feature.

The cases of refractory anemia may be divided into two general groups, those in which the anemia is associated with a recognized disease entity and is presumably secondary, and those in which it seems to occur independently. The secondary type is included because frequently it cannot be recognized from the hematologic data alone and hence it may be a serious problem in differential diagnosis. Of the forty patients with secondary refractory anemia ten had Hodgkin's disease involving the bone marrow, four had obscure malignant neoplasms, twelve had presumptive evidence of degenerative disease of the liver, one had tuberculosis of the bone marrow and thirteen had either lymphoid or myeloid aleukemic leukemia. In many instances conclusive evidence of the primary disease process was obtained only from the histologic study of sternal bone marrow removed at biopsy.

Sixty cases of apparently primary refractory anemia were studied and divided into four groups on the basis of the pathologic changes of the bone marrow as seen at biopsy, at autopsy or both. The four basic pathologic changes were sclerosis, normal cell hyperplasia, immature cell hyperplasia and immature cell hypoplasia. Sclerosis of the marrow was present in three cases. In twelve, an active marrow with apparently normal maturation was present, and in eight of these the presence of a hemolytic process was confirmed by studies of urobilin output. Studies of pigment excretion were not made in the remaining four cases. Hyperplastic, cellular marrows, infiltrated densely with uniformly immature cells, were present in eighteen cases. The cells were so undifferentiated that their nature could not be determined absolutely. This condition has been described by Thompson, Richter and Edsall[1] and is considered by some observers to be aleukemic leukemia. The absence of a leukemic blood picture, of leukemic tumor and of leukemic infiltration of organs is the basis for considering the group to be an independent one. The remaining twenty-seven cases were marked by hypoplasia to almost total aplasia

1. Thompson, W. P.; Richter, M. N., and Edsall, K. S.: Am. J. M. Sc. 187: 77 (Jan.) 1934.

of the marrow structure. This group differed from the one with hyperplastic marrow only in cellularity, since the predominant type is a similar primitive, immature cell. When considered physiologically the condition is the result of disturbance of cell destruction or cell maturation or of both factors. Considerable information is afforded by studies of pigment excretion as an index of cell destruction and of coproporphyrin-I excretion as a measure of marrow activity.

The etiology of refractory anemia is obscure, but certain suggestions deserve consideration. The basic lesion of the bone marrow in the types marked by decrease of cell production with a predominance of primitive cell elements is very like that seen in acute granulopenia, a condition in which an abnormal susceptibility to certain chemical compounds has been established as etiologic in certain instances. Some evidence is at hand which suggests that a similar process if continued sufficiently long may be operative in causing a disturbance of erythropoiesis as well as of myelopoiesis.

Analysis of sixty cases of primary refractory anemia gives a history of exposure to compounds which may be toxic to the hemopoietic system in twenty-seven cases, or 45 per cent. This number is considered to be significant in view of the limited knowledge of the various factors which give rise to drug hypersusceptibility.

No suggestive toxic agent could be established for the group with sclerotic marrow. Two patients with hemolytic anemia and hyperplastic, normal cell marrow had been exposed to benzene and two had been given arsphenamine. One patient with active marrow for whom no pigment studies are available was proved to be sensitive to aminopyrine. Of the patients with hyperplastic immature cell marrow, two had radium poisoning, two had used aminopyrine, one was exposed to a number of potentially toxic solvents in his work as a roofer, two had been exposed to benzene, two had used a potentially toxic hair dye for many years and one had used cresote medicinally. Of the patients with hypoplastic immature cell marrow, four had been exposed to benzene, two to arsphenamine, one to cinchophen, two to aminopyrine, one to acetophenetidin, one to hydroquinone, one to possibly toxic solvents in his work as a printer, two to excessive amounts of a naphthene-containing insecticide, one to creosote and

one to a potentially toxic hair dye. It should be emphasized that in most instances the exposure to possibly toxic chemicals was no greater than most persons can tolerate without difficulty.

The outcome of refractory anemia is not necessarily fatal, although it may well be so. For secondary anemia the prognosis is that of the primary disease. Of the sixty patients with primary anemia, seventeen had a well defined remission at some time. Of the seventeen remissions, fourteen were complete and three were only partial. Of all the remissions, six continued as long as the patients were observed, but in the remainder of the patients the disease recurred and terminated fatally.

Any discussion of the therapy of refractory anemia must be largely negative. Stomach preparations, whole liver and liver extract given in maximum dosage by both oral and parenteral routes have uniformly failed. No iron preparation has had any effect. Vitamin C has failed not only to alter the course of the disease but also to control the hemorrhagic phenomena. Transfusion has never had more than a transient effect on the course of the disease but may carry the patient along until the marrow function has recovered spontaneously. Some evidence is at hand that, for carefully selected patients with ample functioning bone marrow, an elevated reticulocyte level and evidence of definitely increased hemolysis, splenectomy may be a useful procedure. The nucleic acid derivatives have been uniformly without effect.

SUMMARY AND CONCLUSIONS

Analysis of 100 cases of refractory anemia proves the existence of two major groups, those of primary and those of secondary anemia, in which the condition cannot always be differentiated on hematologic evidence. The cases of primary anemia may be subdivided on pathologic grounds into four groups, based on the presence of sclerotic, normally hyperplastic, immature hyperplastic or immature hypoplastic marrow. The physiologic mechanism is a disturbance of cell production or of cell destruction or both. When cell production is interfered with, the basic lesion is a predominance of primitive cell elements. In 45 per cent of the cases of primary anemia, a history of exposure to chemical compounds which may be hematologically toxic was present. Temporary remissions occurred in 25 per cent and permanent remissions in 6 per cent. No treatment was effective.

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BOVINE MASTITIS

III. A COMPARISON OF THE BACTERIOLOGICAL AND PHYSIOLOGICAL REACTIONS OF NORMAL AND MASTITIS MILK FROM YOUNG COWS

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(Received for publication, January 29, 1938)

The present paper has a twofold purpose. First, it is proposed to describe in detail the laboratory methods referred to in our previous publications.[1,2,3] Secondly, the results will be given of the laboratory examination of the foremilk from 8 first-calf heifers before and after their udders were infected with a double zone hemolytic streptococcus.[2,3] While it is not our intention to set up a standard for the reaction of the foremilk in normality or disease on the basis of the few first-calf heifers used, it is conceivable that data compiled from the daily examinations of a small number of cows may be of more value than those from the weekly or monthly examinations of a larger group.

Literature Pertaining to the Diagnosis of Mastitis

Munch-Petersen[4] in 1933 reviewed nearly 2,000 publications concerning mastitis and in his general summary says: "It is very apparent that the criteria accepted by various authors as to the presence or absence of mastitis differ considerably. The desirability of evolving some simple and reliable tests or series of tests which can be relied upon for the diagnosis of this disease needs no stressing, but the accurate evaluation of diagnostic methods is well nigh impossible in the absence of any agreement as to what constitutes mastitis on the one hand and normality on the other. Nowhere in the literature perused in preparation for this summary has the writer found adequate work on this fundamentally important point, and there seems

to be outstanding need for accurate, detailed and often repeated examination of the udders and milk of cows in their first lactation period in order to establish adequate criteria on which normality, or departures therefrom, may be judged. So far as the immediate cause of mastitis is concerned the important part played by streptococci is a point on which practically every author is agreed and although differences of opinion still exist as to the nature of the streptococci involved it appears to be widely recognized that organisms conforming in their main characters to those of *Streptococcus mastitidis* or *Str. agalactiae* are of outstanding importance."

Following the development of a colorimetric method by Baker and Van Slyke[5] for the detection of abnormal milk, wide use has been made of the hydrogen ion determination in the detection of mastitis infections of the udder. Rosell[6] and Udall and Johnson[7] consider it a useful and reliable test. Hucker et al.[8] and Stableforth[9] assert that although positive reactions are generally indicative of mastitis a negative reading does not necessarily indicate that the quarter is uninfected. Plastridge and Anderson[10] found that a positive bromthymol blue reaction was obtained in about 70 per cent of cows that frequently secreted milk of abnormal appearance, but that this test was of slight value in the detection of mild cases of the disease. It is generally assumed that the pH value of normal milk varies between 6.5 and 6.7.

Rosell,[6] Hayden,[11] and Hucker et al.[8] suggest that an increase in the concentration of chlorine in milk signifies an alteration in its character. Rosell states that normal milk should not contain more than 0.14 per cent chlorine, whereas Hucker and his associates suggest that a minimum of 0.16 per cent offers a closer correlation between this test and the presence of physical changes in the udder.

Another valuable test in the detection of udder infection, advanced by Prescott and Breed,[12] is the direct microscopic count of the leucocytes, the number of cells being estimated on the basis of 1 cc. of milk. Hucker and his coworkers[8] maintain that a cell count over 3,000,000 per cc. is generally indicative of past or present infection with streptococci, while Little and Jones[13] so regard a cell count above 1,000,000. More recently Hucker[14] states: "Milk containing more than 500,000 cells per cc. always indicates an abnormal or pathological condition

in the udder. . . .All quarters free from scar or indurated tissues never show demonstrable streptococci or cells in excess of 150,000 per cc. in the milk." He[15] further says: "It is also to be concluded that the presence of 500,000 cells per cc. when milk from individual quarters is examined definitely indicates an abnormal condition in the quarter involved. If a composite sample of all four quarters is examined the presence of a lower cell count may indicate infection in one quarter and in like manner if a can sample is being examined, the presence of 100,000 cells per cc. probably will indicate that milk from an infected quarter is included in the sample being examined." Moreover, Cherrington et al.[16,17] and Prouty[18] suggest that the cell count from normal udders is relatively low, varying from 60,000 to 70,000 or less, and the former authors state that a leucocyte count in excess of 100,000 cells per cc. is generally found in milk from infected quarters.

Various bacteriological methods have been employed in the diagnosis of mastitis by different workers. The usual technique is to dilute the milk with normal saline and transfer 1 cc. of this dilution to a Petri dish to which defibrinated horse or ox blood and agar are added. Frequently the dilution is determined by a standardized loop and the milk is plated undiluted. Many investigators have found that incubated milk or sediment when prepared in a film or streaked over the surface of agar plates or agar slants affords a suitable means of diagnosis.

Before completing this brief review of the literature, it seems desirable to discuss the selection of the sample of milk most suitable for the bacteriological determinations. In the literature pertaining to mastitis it is occasionally stated that the foremilk was used in the examination. In the collection of the sample, however, the first stream or two of milk was discarded to avoid contamination. Since in different cows the size of the teats and their apertures varies greatly,[3] in some animals the first stream or two of milk withdrawn from the udder may empty the canal and lower portion of the cistern. It is therefore questionable whether the majority of such samples of milk really represent the secretion present in the teat.

Furthermore, it is evident from Munch-Petersen's[4] survey on "Selection of Milk Samples" that there exists a great difference of opinion

among investigators as to the portion of milk most suitable for the identification of streptococci from affected quarters. Objection is raised to the use of the foremilk because it may normally contain a greater number of miscellaneous bacteria, which would make identification more difficult, and because the danger of contamination with bacteria outside the teat is greater in foremilk samples. A number of workers, however, found that streptococci were more frequently identified in the foremilk than in the middle portion or strippings.

When foremilk is referred to in this and other papers from this laboratory, it means the first milk drawn from the udder. As already mentioned, our laboratory examinations were confined to the foremilk, but in one experiment the following data were collected for comparative purposes.

A Comparison of the Results of the Bacteriological Examination of Foremilk, Middlemilk, and Strippings

The results of the bacteriological examination of equal amounts (not over 10 cc.) of the foremilk, middlemilk, and strippings from two heifers begun 3 days after inoculation with a hemolytic strain of bovine streptococci are given in Table I.

While streptococci were found in all specimens, the totals indicate that they were more than ten times as numerous in the foremilk as in either of the other samples.

METHODS

Before the collection of milk for laboratory examination, the udder and teats of the cow were wiped thoroughly with a clean damp cloth and dried with another clean cloth. In certain observations the teats were washed with soapy water, dried, and then wiped with a pledget of cotton saturated with 95 per cent alcohol.

The samples of milk were obtained at the morning milking in sterile 50 cc. test tubes or half pint bottles, chilled, and immediately examined in the laboratory. Usually 2 to 5 cc. of the foremilk was used, but occasionally larger amounts were taken.

Hydrogen Ion Concentration.—The colorimetric method was used in determining the pH reaction. The indicators employed were

phenol red, brom cresol purple, and methyl red. In the beginning a method described by Brown[19] was used but later a portion of the milk was distributed into three cups of a Coors Porcelain Plate and the

TABLE I

*The Results of the Bacteriological Examinations of an Equal Amount of Foremilk, Middlemilk, and Strippings from the Right Fore Quarters of Two Cows**

Cow No.	Days after inoculation	Colonies per cc. of milk		
		Foremilk	Middlemilk	Strippings
2017	3, a.m.	11,520	60	200
	4, a.m.	13,440	420	3,200
	4, p.m.	8,960	196	1,560
	5, a.m.	27,520	550	800
	6, a.m.	4,480	2,200	2,800
	7, a.m.	17,280	1,790	1,230
	7, p.m.	12,800	410	700
	8, a.m.	30,720	2,250	8,960
	8, p.m.	12,800	1,650	1,750
	9, a.m.	32,000	1,820	1,100
	9, p.m.	13,440	1,140	1,910
	10, a.m.	60,160	12,160	5,120
2018	3, a.m.	1,000	30	30
	4, a.m.	1,920	130	60
	4, p.m.	21,120	830	1,020
	5, a.m.	43,520	3,840	4,480
	6, a.m.	32,000	3,420	1,700
	7, a.m.	23,040	300	440
	7, p.m.	14,080	360	2,560
	8, a.m.	52,480	860	1,120
	8, p.m.	8,960	950	370
	9, a.m.	8,320	870	370
	9, p.m.	33,920	190	2,560
	10, a.m.	362,000	5,760	22,400
Total of 24 examinations........		847,480	42,186	65,640

* The quarters had been inoculated with a double zone beta hemolytic streptococcus.

indicators added. With this procedure each cup represented the reaction to a single indicator and differences in color could be readily ascertained.

Determination of the Presence of Chlorine.—The method of Rosell[6] is to add 10 cc. of milk to 40 cc. of distilled water containing 8 to 12 drops of a 10 per cent solution of potassium dichromate. To this mixture a 1/10 N solution of silver nitrate is added until the yellow solution assumes a reddish tinge, which indicates the desired end point. The amount of chlorine in such a sample is determined by multiplying the number of cc. of silver nitrate used by 0.335.

Since our samples of foremilk were small, it was necessary to reduce the dilution examined to conform with the amount at hand. Therefore a modification of Rosell's method was developed in which the milk and distilled water were reduced without affecting the reaction. Thus 1 cc. of milk was diluted with 4 cc. of distilled water to which 3 drops of a 10 per cent solution of potassium dichromate had been added. The dilution was then titrated with N/20 silver nitrate.

Leucocyte Count.—The number of leucocytes per cc. was estimated by the direct method of Prescott and Breed.[12] The films, however, when dry were stained with Newman's solution, formula No. 2.[20] In each sample of milk the count represented the total number of leucocytes in 1 cc. rather than the combined count of the leucocytes and fixed body cells.

Bacteriological Examination.—The routine procedure in our laboratory examinations was to culture the foremilk directly, and the final results were always determined in this way. 1 cc. of freshly drawn milk was diluted in 9 parts of sterile normal saline and thoroughly mixed. Then 1 cc. of this dilution was plated with 0.5 cc. of defibrinated horse blood and 12 cc. of a 2 per cent veal infusion agar or digest agar. Occasionally, for comparison, a small portion of the sample was centrifuged and with a loop the sediment was streaked over the surface of a blood agar plate, or suspended in NaCl and plated. In addition, portions of the milk were sometimes incubated for 24 hours and examined culturally. The plates were incubated for 24 to 36 hours at 38°C. In making the bacterial count following incubation a dissecting microscope and a Wolffhuegel counting glass were used. The plates were searched for typical colonies, and transfers when necessary were made to bouillon or blood agar slants to determine the cultural classification of the streptococci.

The veal infusion agar was made according to the usual methods except that the peptone was added to the juice after extraction. In the preparation of the digest agar, 400 gm. each of ground fresh beef hearts and pig stomachs were mixed with 35 cc. of concentrated HCl to which 3000 cc. of distilled water was added. This mixture was placed in a water bath at 50°C for 18 to 20 hours after which the degree of digestion was determined by the Bieuret reaction. The digest was then transferred to agateware receptacles, 10 cc. of N/1 NaOH per liter added, and the mixture brought to a boil over a free flame. After heating, it was poured into pyrex jars and stored overnight in the refrigerator. On the following day the mixture was strained through gauze to remove the fat and other solid material, and approximately 50 cc. of N/1 NaOH added to each liter. The digest was weighed and then boiled for 5 minutes with the adjustment of the pH to 7.0–7.2 by the addition of N/1 NaOH. The loss in volume by boiling was adjusted by adding H_2O. For plating, 2 per cent agar was mixed with the broth which was tubed in 12 to 15 cc. amounts.

The Production of Experimental Mastitis.—The method of inoculation of streptococci into the udders of first-calf heifers has been discussed in detail in previous publications.[1,2,3]

Results of the Daily Laboratory Examination of the Foremilk from Young Cows

Table II gives the results of the examination of the milk from 31 quarters of 8 first-calf heifers before the inoculation with hemolytic streptococci.

It will be noted in Table II that the majority of the 1010 daily samples of the foremilk showed a pH of 6.6 and that the chloride determinations were not above 0.135 per cent. The leucocytes were not over 300,000 in 95 per cent of the samples and were absent in the great majority. In 5 per cent of the samples the leucocyte count ranged from 300,000 to one million cells or over. The total bacteria count, in 83 per cent of the daily examinations, was not over 1000 colonies per cc. of milk; in 70 per cent the count was under 500.

TABLE II

The Results of the Daily Laboratory Examination of Milk from Separate Quarters of First-Calf Heifers before Their Udders Were Inoculated with or Exposed to a Hemolytic Streptococcus

No. of cows	No. of quarters	No. of examinations	pH		Chloride determination		Direct leucocyte count per cc. of milk						Total bacteria count per cc. of milk			
			6.6	6.7 and over	0–0.135	0.135 and over	0 cells	Up to 75,000	75,000–300,000	300,000–500,000	500,000–1,000,000	Over 1,000,000	0–500	500–1,000	1,000–5,000	Over 5,000
8*	31	1010	1003	7	1004	6	779	99	82	11	26	13	711	128	119	82
Percentage.............			99.3	0.7	99.3	0.6	77	9.8	8.1	1.1	2.6	1.3	70.5	12.7	11.8	5.1
							95			5			83		17	

* First-calf heifers Nos. 1919, 1927, 1943, 1945, 1951, 1962, 1972, and 1973[2,3].

TABLE III

*The Results of the Laboratory Examination of Milk from Subclinical Cases of Hemolytic Streptococcic Mastitis**

No. of cows	No. of quarters	No. of examinations	pH			Chloride determination				Direct leucocyte count per cc. of milk								Total bacteria count per cc. of milk					
			6.6	6.7	6.8 and over	0–0.135	0.135–0.140	0.140–0.150	0.150 and over	0–75,000	75,000–300,000	300,000–500,000	500,000–1,000,000	1,000,000–2,000,000	2,000,000–5,000,000	5,000,000–10,000,000	Over 10,000,000	10–3,000	3,000–10,000	10,000–20,000	20,000–40,000	40,000–100,000	Over 100,000
	31	2163	1016	744	403	1204	219	242	498	49	72	82	242	375	677	297	369	378	995	419	196	132	43
Percentage......			47.0	34.4	18.6	55.6	10.1	11.2	23	2.3	3.3	3.8	11.2	17.3	31.3	13.7	17.0	17.5	46.0	19.4	9.1	6.1	2.0
			81.4			65.8		34.2		5.6		94.5							65.4				

* The examinations of the milk made between the first inoculations or exposures and the onset of mastitis have been omitted from this table.

Results of the Daily Laboratory Examination of the Foremilk from Subclinical Cases of Mastitis

In Table III the results are submitted of the examinations of the milk following the onset of mastitis in quarters either inoculated with or exposed to hemolytic streptococci.

In 2163 daily examinations, 81 per cent of the samples gave a pH reading of 6.6 to 6.7. In 55 per cent the chloride content was not above 0.135 per cent and in 10 per cent it varied between 0.135 and 0.140. The pH values in 18 per cent of the samples was 6.8 or above, and the chloride content in 34 per cent was over 0.140. Therefore, with these two critical tests the determinations in only 52 per cent of the samples were indicative of udder abnormality.

In the daily examination of the foremilk from subclinical cases of experimental mastitis the most striking differences were encountered in the bacteriological examination and the direct leucocyte count. Typical hemolytic streptococci were detected on each daily examination and in only 17 per cent of the days was the total count below 3000 colonies per cc. The bacteria count in 65 per cent of the examinations varied between 3000 and 20,000 colonies per cc. When these higher counts were encountered in the blood agar plates the streptococci were usually present in pure culture; whereas with a bacteria count under 1000, micrococci and other miscellaneous organisms also developed. The leucocyte count in 94 per cent of the examinations varied between 300,000 and 10,000,000 cells or over.

DISCUSSION

In 1010 daily examinations of the foremilk from 8 normal first-calf heifers, most of the determinations were within the limits generally accepted for normal milk. After the onset of subclinical mastitis in these 8 animals, 2163 daily examinations of the foremilk showed that the bacteriological plating of the milk in blood agar was more efficient in the detection of infection than any other method employed, for typical hemolytic streptococci were observed in the milk on every examination. Since the streptococci used in this study produce characteristic double zones and orange pigmentation in blood agar,

their identity was established without difficulty. In over half of the examinations the total bacteria count per cc. of milk varied between 3000 and 20,000, which further shows that in the early subclinical infections with this strain of streptococcus the organisms usually are not present in the foremilk in relatively large numbers.

The next most reliable test was the direct leucocyte count; in 94 per cent of the examinations the count per cc. of milk was between 300,000 and 10,000,000 cells or over. In the normal foremilk the reverse was found, since 95 per cent of the samples had between 0 and 300,000 cells per cc. While such a leucocyte count may be considered normal for the foremilk from first-calf heifers early in their first lactation periods, other examinations have shown that in older cows which so far as can be determined are normal, the foremilk has occasionally a count between 500,000 and 1,000,000 cells per cc.

The results of the hydrogen ion and chloride determinations showed that these two tests were of less value in the identification of early subclinical infections than the bacteriological plating of the milk and the direct leucocyte count. These findings are in agreement with those of Shaw and his associates,[21] that the tests for the determination of the hydrogen ion concentration and the percentage of chlorine are not reliable in the detection of chronic mastitis.

SUMMARY

A review of the daily laboratory examination of the foremilk from 31 quarters of 8 young cows before and after infection with streptococci is submitted.

The results of 1010 daily examinations of the foremilk from these animals before infection show that most of the determinations were within the limits generally accepted for normality.

After the production of a subclinical mastitis, 2163 examinations of the foremilk indicate that the plating of the foremilk in blood agar and the direct leucocyte count are more efficient in the detection of infection than either the hydrogen ion or the chlorine test.

The author is indebted to Mr. Edward J. Foley for valuable assistance in this study.

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TOXEMIA OF PREGNANCY IN THE RABBIT

II. Etiological Considerations with Especial Reference to Hereditary Factors

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A description of the clinical manifestations and pathological lesions of toxemia of pregnancy in the rabbit and evidence indicating an analogy with eclampsia in man were presented in a previous paper (1). Clinical and pathological study suggested that the disorder was of endogenous origin, but a discussion of etiological factors proper was deferred pending presentation of the evidence derived from a study of the incidence.

The object of the present paper is to analyze the incidence of the disorder with respect to pregnancy, age, breed and genetic constitution, and to consider the results of this study together with the clinical and pathological findings as factors of etiological significance. Detailed analysis of the incidence will be limited to a period extending from November, 1935, to December, 1936, during which there was an unprecedented number of cases, but the previous occurrence of the disorder will be described and factors of possible significance in the genesis of the outbreak will be discussed.

Materials and Methods

The material for the present report is based on 72 fatal cases of toxemia which occurred between November, 1935, and December, 1936, supplemented by data obtained from previous observations. The colony in which the disorder occurred and the composition of the population were described in the first paper of this series (1). The origin, integrity and relations of the different pure breeds have also been described in previous publications (2, 3).

Incidence

Toxemia of pregnancy in the rabbit may occur as a rapidly fatal affection or as a comparatively mild, frequently asymptomatic disorder followed by recovery. The detection of non-fatal cases is not always possible, and as the incidence cannot be accurately determined, analytical studies are necessarily limited to the occurrence of fatal cases.

Fatal toxemia of pregnancy was first noted in 1931 and two cases were recorded during that year (Table I). The number of cases gradually increased in following

TABLE I

The Annual Incidence of Toxemia from 1931 to 1936

Year	Number of cases of toxemia
1931	2
1932	3
1933	7
1934	11
1935	25
1936	55

TABLE II

The Monthly Incidence of Toxemia during the Outbreak Period

	1935		1936											
	Nov.	Dec.	Jan.	Feb.	Mar.	Apr.	May	June	July	Aug.	Sept.	Oct.	Nov.	Dec.
Number of cases of toxemia	0	17	8	19	7	4	5	3	0	1	1	2	4	1

years, but the incidence remained relatively low until in November, 1935, a rapid increase began and continued through December, 1936. The monthly incidence during this outbreak period is presented in Table II. It is of interest, in view of the seasonal variations in the frequency of human eclampsia, that the majority of cases both during this period and in previous years occurred during the late winter and early spring.

72 animals died of the disorder in this outbreak, an incidence in the female population of 11.07 per cent. Cases were not distributed evenly throughout the population, but occurred with greater frequency in certain breeds and families, and in the following paragraphs the incidence during the 13 months' period will

be analyzed with reference to the differential response of various elements of the population.

It should be emphasized from the beginning, however, that all susceptible animals were not affected by the disorder during the period under consideration. A number have since died of toxemia and others will undoubtedly die at future dates. The results obtained from analysis of the incidence are, therefore, not absolute but relative to that period only, and differences in the incidence in various constitutional groups would probably be greater if the period were longer or equalled the life of the population. The occurrence of the disorder in both sporadic and outbreak form suggests that the incidence may be greatly influenced by environmental factors and that under favorable conditions a susceptible animal may live its life unaffected. Therefore, as the only test of susceptibility is death from toxemia, susceptibility studies must necessarily be conducted during periods of high incidence although such periods may include only a short portion of the life of the population.

Relation to Pregnancy

The disorder was not limited to pregnant rabbits, but occurred postpartum and in resting animals. In the present outbreak 59.3 per cent of cases were in pregnant does, 20.8 per cent in postpartum and 19.4 per cent in resting females.

Among pregnant does all cases with one exception occurred during the last week of gestation, usually on the 28th day. The one exception occurred on the 17th day of pregnancy. 5 cases of the disease occurred on the 1st day following delivery and only 2 cases appeared later than a week postpartum.

With reference to deaths among resting females, it is of considerable interest that at autopsy corpora lutea were found in the ovaries. 12 of these animals had recently been mated and were presumably in a condition of pseudopregnancy. In explanation of this term, it should be pointed out that in the rabbit ovulation is induced by côpulation, and if fertilization fails to occur, the female may still exhibit all the symptomatic changes of pregnancy. In such animals the temperamental changes associated with pregnancy have frequently been observed and nest building and lactation are not uncommon near the end of the pseudogestation period which is of irregular duration. These manifestations, however, are not limited to infertile mated animals, but are also occasionally noted in unmated animals. In two instances the typical clinical symptoms and pathological lesions of thė disorder were found in animals that had not been mated in over 6 months but had been caged in open wire compartments in close proximity to other animals. It seems to be well authenticated that rabbits may ovulate as a result of contact without copulation, and such instances indicate that mere proximity to other animals may be sufficient stimulus to induce ovulation and that pseudopregnancy of this order may be of common occurrence.

The duration of the state of pseudopregnancy in does dying in that condition is difficult to determine inasmuch as many animals had been mated a number of times in the period preceding the occurrence of the disorder. When estimated from the last mating, the period averages 10 days, but if only those cases are considered in which a single mating had been made, the period averages 25 days, and this approximates that found in true pregnancy.

Multiparity.—There were 66 fatal cases of the disorder in multiparous does, an incidence of 10.3 per cent, and only 6 cases, or an incidence of 2.6 per cent, in primiparae. The disorder did not occur in virgin females. This distribution is contrary to that observed in human eclampsia where the frequency is far greater in primiparae than in multiparae. It should be noted, however, that in the rabbit repeated attacks of toxemia occur and the primary attack may be mild and asymptomatic. It is probable that the disorder occurs in primiparous rabbits with far greater frequency than is recognized and that the higher incidence of fatal cases in multiparae is due to repeated attacks.

Fertility.—In the routine conduct of the colony, animals are examined for pregnancy on the 10th day after mating and if found non-pregnant, are remated. The fertility of the colony is thus under constant check, and examination of the records of animals that subsequently died of toxemia brings out a point of interest.

The multiparous does had borne from 1 to 10 litters each with an average of 5 litters. 12 of these animals had gone through uncomplicated pregnancies from 1 to 3 months previous to the fatal attack, but the remaining 54 animals had not borne litters for periods ranging from 7 to 15 months. This long interruption was due in part to cessation of breeding during the summer of 1935, but in larger part to infertility for the animals had been mated repeatedly without conception, both before and after the summer interval. A similar history of sterility obtained in the primiparae and in one instance as many as 12 non-fertile matings had been made. The frequent history of a long period of unproductivity followed by resumption of fertility and death from toxemia in the ensuing pregnancy may be of etiological significance.

Age

The age incidence of fatal cases of toxemia varied from 5 to 53 months. On a percentage population basis the incidence was 2.4

per cent among animals less than 1 year old, 17.8 per cent in the group between 1 and 2 years, 15.5 per cent in the group between 2 and 3 years, 22.2 per cent in the group between 3 and 4 years, and 18.1 per cent in the group between 4 and 5 years.

TABLE III

Distribution of Cases of Toxemia in Pure Bred Animals and in Various Hybrid Generations

Breed	Pure bred		First hybrid generation		Second hybrid generation		Backcross hybrid generation		Other hybrid generations		Total hybrids		Total hybrid and pure bred	
	Number of animals	Mortality	Number of animals	Mortality	Number of animals	Mortality	Number of animals	Mortality	Number of animals	Mortality	Number of animals	Mortality	Number of animals	Mortality
		per cent		*per cent*		*per cent*		*per cent*		*per cent*		*per cent*		*per cent*
Belgian	31	3.2	32	3.2	0	0	9	0	54	7.4	95	5.2	126	4.7
Blue Beveren	3	0	3	0	1	0	9	11.1	6	0	19	5.2	22	4.5
Dutch	19	26.3	23	21.7	7	14.2	18	5.5	37	13.5	85	12.9	104	15.3
English	42	14.2	49	6.1	1	0	7	14.2	100	10.0	157	8.9	199	10.0
Himalayan	14	0	19	42.1	2	0	2	0	15	13.3	38	26.3	52	19.2
Havana	31	9.7	46	21.7	4	25.0	3	33.3	59	13.6	112	17.8	143	16.0
Polish	4	50.0	57	33.3	11	9.0	9	0	34	14.7	111	22.5	115	23.4
Rex	2	0	15	26.6	4	25.0	15	20.0	17	5.9	51	17.6	53	17.0
Chinchilla, Lilac, Marten, French Silver, Tan, Sable	15	0	21	0	4	0	7	0	18	0	53	0	68	0
Himalayan-Albino hybrids									115	9.5	115	9.5	115	9.5
Polyhybrids of complex genetics									31	3.2	31	3.2	31	3.2
Total............	154	11.03	181	16.4	23	13.0	82	9.7	209	6.7	496	11.08	650	11.09

The low incidence noted in the youngest group was probably related to parity rather than to age which was apparently of little significance in the determination of susceptibility.

Breed

The distribution of fatal cases of toxemia in the pure bred animals and in the various hybrid generations of each breed is shown in Table III. The hybrids are classified into first, second and backcross generations, and a fourth class designated as other generation hybrids includes all other animals derived from the pure

breeds with the exception of two groups which are listed separately. One of these is composed of polyhybrid animals of extremely complex genetic origin; the other is a group of Himalayan-Albino hybrids that had been inbred since 1918. The Himalayan ancestors of this group were pure bred animals, but the Albinos were a mongrel stock of uncertain origin derived in part from a Dutch-Polish cross. All other hybrids are listed under each of the various breeds from which they were derived and, as this necessarily involves duplication, the total number of animals included in each generation is listed in the final column of the table.

There was no significant difference in the incidence of the disorder in pure bred and hybrid animals; the incidence was 11.03 per cent in pure bred stocks and 11.08 per cent in hybrids. There were, however, marked differences in the distribution of cases in the various pure breeds and in the different hybrid classes and subgroups.

Pure Breeds.—The disorder did not occur in the Beveren, Chinchilla, Himalayan, Lilac, Marten, Rex, Sable, French Silver or Tan breeds. In the remaining breeds the incidence was highest in the Polish and Dutch, intermediate in the English and Havana, and lowest in the Belgian.

A number of breeds were represented by relatively few animals, and the significance of the incidence is doubtful if measured statistically. Other evidence, however, shows that with two exceptions, the pure bred animals present during the outbreak period were truly representative of their breed and the results obtained may be considered as indicative of the relative incidence had larger groups been available. The two exceptions consist of the Himalayan and Rex breeds. Typical cases of toxemia occurred in both of these breeds in years previous to the period under consideration and occurred during the present outbreak in animals derived from repeated backcross matings, and it is assumed that the small pure bred groups present in the colony were not representative.

In view of the wide differences in incidence breed may, therefore, be regarded as a factor of considerable importance in the determination of susceptibility. It is of interest in this connection that the Dutch, Polish and Havana breeds, all of which showed high susceptibility, are racially related and belong to a group which is fundamentally Dutch.

Hybrids.—The incidence in Himalayan and Rex hybrids was high, but otherwise the position of the different hybrid groups was generally comparable with the arrangement of their pure bred ancestors. With the exception of the Himalayan, Rex and Beveren groups, the hybrids derived from breeds in which toxemia did not occur were also un-

affected by the disorder. The position of the Himalayan and Rex breeds in this respect has been referred to above. The single case in the Beveren hybrids occurred in an animal obtained from a backcross mating of an Havana-Beveren hybrid, and the factors determining susceptibility may have been carried over from the Havana breed.

The relatively low frequency of toxemia among Himalayan-Albino hybrids is of interest in view of the fact that in former years the highest incidence occurred in this group. It is probable, however, that the majority of susceptible animals had previously died of the disorder, and that the population present during the outbreak was to a large extent composed of non-susceptible survivors.

On a basis of the incidence in the various pure breeds and their hybrid derivatives, the arrangement of breeds as factors in the determination of susceptibility stands Polish, Himalayan, Rex, Havana, Dutch, English, Belgian, Beveren in order of decreasing importance. Additional and more detailed information of the relative importance of breed in this connection may be obtained by further analysis of the first hybrid generation and a classification of animals with reference to both parental lines.

First Generation Hybrids.—41.6 per cent of all cases of toxemia occurred in first generation hybrids and the incidence in this group was 16.4 per cent compared with an incidence of 8.9 per cent in the remainder of the colony.

There were marked differences in mortality in the F_1 hybrids derived from different breeds, but the position and arrangement of the various groups in an incidence scale is in general agreement with that noted in total hybrids in the previous table.

No cases occurred in the first generation progeny of the Beveren, Chinchilla, Sable, French Silver or Tan breeds. The incidence was greatest in the groups derived from the Himalayan, Polish, Rex, Havana and Dutch breeds and least in those derived from the English and Belgian breeds.

The F_1 generation hybrids may be subdivided into two classes for more detailed examination. One class which was derived from the mating of pure bred animals with unrelated hybrids and in a strict sense is not a true F_1 generation, contained 86 animals of which 8 died of toxemia. The other class was derived from the crossing of pure breeds and contained 100 animals of which 22 died of the disorder. Closer analysis of the first class with its more complex breed relationships fails to give further information concerning the influence of breed on the incidence of the disorder. The animals of the second class, however, may be analyzed with

reference to both parental lines as in Table IV, and by comparing the incidence in vertical and horizontal planes the relative influence of each breed on the susceptibility of the F_1 generation may be gauged.

21 separate groups of first generation hybrids result from such a classification, but cases of toxemia occurred in only 8 of these groups, namely, the Dutch, English, Himalayan and Havana-Polish hybrids, the Belgian, English and Rex-Havana hybrids, and the Himalayan-Rex hybrids. These groups were represented by 60 animals and the remaining 13 groups composed of 40 animals were without mortality. It is significant that 22 cases of the disorder or more than 30 per cent of the total incidence occurred in approximately one-tenth of the total female population of the colony.

TABLE IV

Distribution of Cases of Toxemia in First Generation Hybrids (Mortality Per Cent)

	B	D	E	H	HA	P	R	BA	C	M	S	SA	T
B			0		33.3	0							
D			0		0	33.3							0
E	0	0			16.6	33.3		0	0			0	0
H						46.6	25.0						
HA	33.3	0	16.6			35.7	60.0					0	0
P	0	33.3	33.3	46.6	35.7								
R				25.0	50.0								
BA			0										
C			0										0
M											0		
S										0			
SA			0		0								
T		0	0		0				0				

B = Belgian; D = Dutch; E = English; H = Himalayan; HA = Havana; P = Polish; R = Rex; BA = Blue Beveren; C = Chinchilla; M = Marten; S = Sable; SA = French Silver; T = Tan.

The 8 groups were derived from the crossing of 7 pure breeds, all of which with the exception of the Rex and Himalayan breeds contained cases of toxemia. On the other hand, the disease did not occur in the 13 groups derived from the crossing of non-susceptible breeds and of non-susceptible with susceptible breeds.

Examination of the table shows that the various first generation hybrids shared the susceptibility differences exhibited by the parental pure breeds and that, with minor variations in position, the incidence scale of the first generation agrees with that of the pure breeds. Moreover, with the further exception of the Rex and Himalayan hybrids, the proportion of groups affected by the disease in the different first generations varied with the position of the parental breed in the incidence

scale. Thus, cases of toxemia occurred in 4 of the 5 Polish and Havana groups, in 2 of the 4 English groups, and in 1 of the 3 Dutch and Belgian groups. The occurrence of the disorder in both of the 2 Himalayan and Rex groups is considered as additional evidence that the pure stocks of these breeds present during the outbreak were not representative. Evidence that breeds higher in the incidence scale exerted a greater influence on their first generation hybrids is brought out by a comparison of the incidence in the different groups derived from the same breed. For example, the incidence was greater in pure bred Polish than in pure bred Havanas, and the susceptibility of Polish-English and Dutch hybrids was greater than that of Havana-English and Dutch hybrids. In connection with the high susceptibility of Polish hybrids, it should be noted that the incidence of the disorder was greater in the first generation than in the parent breed in all instances with the significant exception of the Polish breed in which the incidence was greater than in any of its first generation hybrids.

Other Generation Hybrids.—The highest incidence in all classes of animals occurred in the first generation hybrids, but in the second and backcross generations the frequency of cases was decreased and approached that noted in the pure bred population.

The number of animals in the second and backcross generations was small and the position of the hybrid groups was not constantly related to that of the breeds from which they were derived. The arrangement of groups in other hybrid generations and in total hybrids, however, was remarkably constant and was in general agreement with that of the parent pure breeds.

It is evident from the data presented above that factors influencing susceptibility were closely associated with breed or race and that the differing susceptibility characteristics of the parent breeds were transmitted to and expressed in their hybrid daughters. The high incidence in the first generation suggests that these factors were complimentary in action, and the increased susceptibility of hybrid groups derived from the more susceptible breeds, together with the relatively low incidence in animals obtained from the crossing of less susceptible breeds is contributory evidence to this effect. The lowered incidence in second and backcross generations, on the other hand, indicates the expected reassortment of characters with an approach to the status of pure breeds.

Constitutional Variation

In previous paragraphs, emphasis has been placed on the marked differences in susceptibility noted in various pure bred and hybrid

groups and the question arises as to whether such differences were referable to racial distinctions or to other constitutional factors incorporated in the stock by chance association. The genetic constitution of the colony has been under investigation for a number of years and the majority of the breeds are known to carry detrimental variations. In many instances pure bred and hybrid transmitters and non-transmitters have been isolated by breeding tests and the comparative importance of the genetic variation and of race as factors in the determination of susceptibility may be gauged.

Examination of Table III shows that the highest incidence in all the various groups of pure bred animals and their hybrid derivatives occurred in the Polish in which there were 27 cases of toxemia, or 37.5 per cent of the total incidence. The animals of this group had been bred largely for study of an hereditary variation characterized by a dwarfing effect which in homozygous form is lethal and produces a miniature individual approximately one-third the size of its normal sibs. Heterozygous animals are approximately two-thirds the weight of their normal sibs at birth, never attain an equal stature and are subject to a variety of functional disorders (4).

68 of the 115 pure bred and hybrid Polish had been found by breeding tests to be transmitters of this variation. There were 18 cases of the disorder in this group, an incidence of 26.4 per cent, in contrast to an incidence of 19.1 per cent in non-transmitters of the same derivation, or an incidence of 9.2 per cent in all non-transmitters of the colony.

One line of our Dutch breed was known to transmit a cretinoid abnormality and 71 animals had been bred from this line for study of the variation (5). 14 of these died of the disorder, an incidence of 19.7 per cent, which is significantly different from an incidence of 6.06 per cent in animals derived from other branches of this breed, or of 10.0 per cent in the remainder of the colony.

The cretinoid abnormality is apparently a genetic syndrome which may be split into its various parts by breeding, and different features of the disorder may be transmitted and inherited independently, while the variation is expressed in its typical form only when its various component parts are recombined in an individual. Breeding tests had shown that the typical variation occurred in the litters of 45 of the 71 animals while the young obtained from the remaining 26 showed only suggestive changes or were entirely normal. The disorder occurred in 15.5 per cent of the first group while in the latter group the incidence was 26.9 per cent. In view of the marked difference in incidence it would appear that transmitters of the entire complex were less susceptible than other animals of the class. It should be emphasized in this connection that numerous breeding tests are necessary to determine the genetic status of an individual, and it is possible that animals classified as partial or non-transmitters died before adequate tests had been made. On

the other hand, it may be that the accessory factors present in transmitters of the entire complex give rise to a constitutional change which alters susceptibility. A complete interpretation of this apparent paradox is not possible on a basis of the present knowledge of the genetics of the variation, but the latter view is given support by the fact that the incidence among hybrid transmitters of both the dwarf and cretinoid abnormalities was 12.5 per cent which is significantly less than the incidence among transmitters of the dwarf variation alone.

The influence of the lethal dwarfing factor in the determination of the susceptibility of pure bred and hybrid Polish is evident, but the relatively high incidence in non-dwarf transmitters of Polish extraction shows that other factors unrelated to this variation and associated with breed were also of considerable importance in this respect. It may be significant that the majority of this group were first generation hybrids. In other breed crosses the first generation approaches the larger breed in size, but the reverse obtains in Polish crosses and such animals are of small stature. It is not improbable, therefore, that the Polish, the smallest of all breeds, carries a dominant dwarfing factor which is expressed in the first generation hybrids and, like the lethal dwarf character, tends toward increased susceptibility.

The presence of the factors concerned in the cretinoid abnormality was also associated with increased susceptibility. The majority of our pure bred and hybrid Dutch carried these factors, but in the few lines in which the abnormality did not occur, the incidence of the disorder was less than the general level of the population. It is apparent, therefore, that the high susceptibility of these animals was not primarily a function of breed factors as in the Polish, but on the other hand, was associated with an hereditary variation incorporated in the majority of the stock.

All deaths in Rex hybrids occurred in a line known to transmit a deformity of the foreleg resembling rickets, a cystic lymphatic enlargement and a blood condition resembling von Jaksch's anemia. In addition, the single case in pure bred Belgians and the majority of cases in Belgian hybrids were in a line that transmitted a lethal metabolic disorder.

Studies of the pathogenesis of the dwarf and cretinoid abnormalities are not complete, but the facts at hand indicate that both are of hypophyseal origin. Moreover, there is evidence that the Belgian and Rex variations also arise from primary endocrine abnormalities.

Cases of the disorder also occurred in breeds and groups of animals that showed or transmitted other physical or functional variations, but analysis showed no significant differences from the incidence in non-transmitters or normal animals, and it is assumed that the susceptibility of these groups was a function of racial factors.

Parent-Progeny Relations

A more detailed examination of the incidence of toxemia with particular reference to the parental rather than the racial relationships

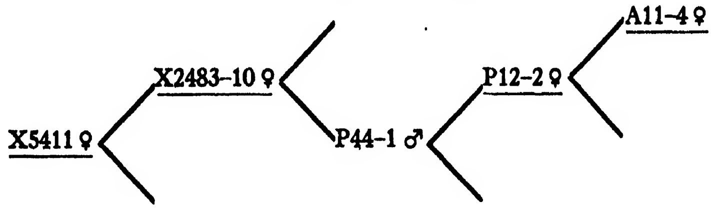
of affected animals brings out additional information regarding the nature of susceptibility.

Analysis of the incidence from this point of view limits the occurrence of the disorder to a relatively small segment of the population, composed in large part of related animals. The total female population of 650 animals had been bred from 140 male and 298 female parents, but the incidence of toxemia was confined to the progeny of 37 of the males and 56 of the females. All cases occurred in animals derived from 62 different matings of these parents, and there were 143 females of this class present in the colony during the outbreak. Thus, on a basis of parental relation the incidence was limited to 22 per cent of the female population of which one-half or 50.3 per cent died of the disorder.

The degree of relationship within this group is indicated by the fact that although the majority of animals were more than four generations removed from the foundation stock of the colony, only 33 males and 39 females of this original stock were concerned in their derivation. It follows that many of the affected

TABLE V

Pedigree Chart Illustrating the Occurrence of Toxemia in Succeeding Generations



Underlined animals died of toxemia.

animals were related through one or both parents. In 5 instances toxemia occurred in full sisters with an incidence ranging from 50 to 100 per cent in the different family groups. 24 animals derived from 9 different females died of the disorder and 45 cases occurred in the progeny of 10 males. In 1 instance 13 affected animals had been derived from a single male and 7 others had been sired by its son.

The disorder was also of frequent occurrence in relatives other than full or half sisters, and pedigree charts of affected animals rarely fail to show additional cases. A simplified pedigree chart illustrating the occurrence of toxemia in succeeding generations is shown in Table V. In 9 instances cases occurred in mothers and daughters during the outbreak while the mothers of 2 other animals had died of toxemia in previous years. In addition, the maternal grandmothers of 9 and the paternal grandmothers of 8 of the affected animals died of the disorder while more remote maternal parents and less immediate female relatives of these and other animals were also frequently affected.

It is clear from the evidence presented above that family as well as race was of considerable importance in the determination of susceptibility. A consideration of immediate parental relations places still greater emphasis on the influence of hereditary predisposing factors.

While classification of the female population into first, second and backcross generations for genetic study is not practicable, some information relative to these factors may be obtained from a differential analysis of the various parent-progeny classes. It should be pointed out, however, that in many instances parents were

TABLE VI

Distribution of Cases of Toxemia Based on Parent-Progeny Relations

	Sons of toxemic doe				Other males				Total	
	Toxemia in progeny (4)		No toxemia in progeny (9)		Toxemia in progeny (40)		No toxemia in progeny (87)			
	No.	Deaths	No.	Deaths	No.	Deaths	No.	Deaths	No.	Deaths
Doe died of toxemia										
Toxemia in progeny (10)	7	1			21	10	8	0	36	11
No toxemia " " (15)	2	0	4	0	19	0	25	0	50	0
Daughter of toxemic doe										
Toxemia in progeny (8)	6	1			17	8	6	0	29	9
No toxemia " " (20)	4	0	4	0	17	0	16	0	41	0
Other females										
Toxemia in progeny (46)	11	8			120	44	20	0	151	52
No toxemia " " (189)	21	0	16	0	136	0	170	0	343	0

Numbers in parentheses represent the number of animals in the various classifications.

not present in the colony during the outbreak period and their status with reference to toxemia was determined at a time when the disorder was sporadic in occurrence while the incidence in the progeny was gauged during an "epidemic" phase. Moreover, many of the affected animals were first generation hybrids and their breeding was designed rather to test their genetic constitution than to supply stock for the colony. As a result, there were relatively few mature progeny present during the period under consideration. In addition it is known that a number of animals of this class as well as of other classes survived the outbreak and have since died with typical clinical and pathological changes. The available data are thus unsuitable in many ways for investigation of the inheritance of susceptibility

but it is improbable that more adequate material will become available in the future, and as the nature of the disorder prohibits an experimental approach to the problem, the facts at hand are presented in Table VI.

Interpretation of these data is largely conjectural in view of the nature of the material and the small classes formed by detailed analysis. Certain findings, however, are definitely suggestive of the scheme of inheritance. It will be noted that approximately the same incidence of one out of three obtained in the progeny of the three classes of females known to transmit susceptibility regardless of their relationship to the disorder. Moreover, in a number of instances the disorder did not occur in the progeny of daughters of toxemic mothers although these daughters were mated with males known to transmit, and it must, therefore, be assumed that their mothers were not homozygous but were heterozygous for susceptibility factors. It follows that other transmitting females were genetically similar. On the other hand, the fact that cases of toxemia occurred in the progeny of all the sons of toxemic mothers when these sons were mated with known transmitters indicates that some transmitting males may be homozygous. The high incidence in the progeny of certain males previously noted offers contributory evidence to this effect.

Conditions of the Colony in Relation to the Present Outbreak

The high susceptibility of certain genetic groups of animals has been a characteristic feature of this outbreak, but in the past many animals belonging to these groups have been under observation and there have been very few deaths that could be attributed to toxemia. It is apparent, therefore, that the present outbreak cannot be ascribed to the susceptibility of that stock alone. On the other hand, the unusual behavior of the colony as a whole suggests the operation of an extraordinary set of environmental factors which may have been of importance in the genesis of the outbreak.

Disturbances in reproduction were widespread throughout the colony during the outbreak period and began in the spring of 1935. During this season the fertility curve usually reaches a peak and frequently 100 per cent of matings are fertile, but in the spring of 1935 this rise failed to occur and the fertility rate fell below that of the preceding winter. The low fertility continued throughout the outbreak period and the percentage of fertile matings decreased from an average normal rate of 60 to 70 per cent to a rate of approximately 37 per cent. In addition, instances of cannibalism, desertion and other manifestations of poor maternal care were unusually numerous and the incidence of dead born litters and of monsters in these litters was markedly increased.

Other manifestations of disturbed reproductive function were encountered in the colony and are of special significance because of their previous rarity. The

diagnosis of pregnancy by palpation on the 10th day after mating has been found from long experience to be 100 per cent accurate, but during the present outbreak there were cases in which pregnancy had been diagnosed on this date and on reexamination a few days later the product of conception had disappeared from the uterus. This abnormality frequently recurred in subsequent matings of the same animal, and throughout the year there were 52 instances of this kind in 39 animals. It was thought that the resorption of feti might be associated with the deficiency of vitamin E, but the disturbance recurred despite the addition of wheat germ oil to the diet of the animals.

In addition the incidence of uterine tumor was increased from an average of about three or four a year to approximately 40 during the outbreak period. The tumors were observed in many breeds and hybrid groups, but were particularly frequent in lines of animals in which toxemia occurred. The clinical history and changes found at autopsy in organs of the endocrine system indicate a correlation between irregularity of function and tumor development, and the prevalence of the tumor during the outbreak of toxemia is suggestive of an etiological relationship.

Other manifestations of physiological imbalance were observed in both the male and female population of the colony. The susceptibility to snuffles, a contagious upper respiratory disease in the rabbit, was not markedly altered as gauged by the incidence of nasal discharge. There occurred, however, a change in the manifestations and locus of the disease as shown by the widespread occurrence of abscesses in both internal and external organs which on bacteriological examination showed pure cultures of organisms generally associated with snuffles.

The exact relationship of this series of disturbances and disorders to the outbreak of toxemia is not clear, but it seems likely that some relationship exists, and it is significant that these disturbances were rarities while cases of toxemia were sporadic but occurred in markedly increased numbers when toxemia became epidemic in incidence.

DISCUSSION AND ETIOLOGICAL CONSIDERATION

The similarity of the clinical and pathological manifestations of eclampsia in man and toxemia of pregnancy in the rabbit was noted in a previous paper. Other points of similarity are brought out by a comparison of the incidence of the two disorders and the available evidence indicates that one is a generic variation of the other.

The occurrence of toxemia in both sporadic and outbreak form is a characteristic epidemiological feature. The variations in incidence of eclampsia are apparently not as pronounced, but Williams states that in his experience "it often happens that months elapse without

the occurrence of a single case when suddenly a number are observed in quick succession" (6). Harrar (7) in a study of the occurrence of eclampsia over a 10 year period in the New York Lying-In-Hospital found an increased frequency during the late winter and early spring, and it is significant that the same seasonal distribution occurs in the rabbit, both during sporadic and outbreak periods. It is worthy of note in this connection that a disorder of pregnant guinea pigs, clinically and pathologically identical with toxemia in the rabbit, shows similar variations in incidence (8).

The frequency of eclampsia is greatest in primiparous women while in the rabbit the highest incidence of fatal cases is in multiparae. There is considerable evidence, however, that the first attack of the disorder in the rabbit may be mild or asymptomatic and that death may occur as the result of a repeated attack in a subsequent pregnancy.

The clinics of different countries report varying incidences of eclampsia (7). Lichtenstein reported 400 cases of eclampsia in 14,836 labors in Leipsic, an incidence of 2.68 per cent; Williams found 110 cases in 11,000 labors in Baltimore, an incidence of 1.0 per cent; while Reinburg noted only 90 cases in 26,511 labors in Paris, an incidence of 0.34 per cent. These differences are statistically significant and suggest that racial factors may be of importance in the determination of susceptibility.

The influence of race as a predisposing factor in the rabbit has been noted. Genetic constitution, apart from essential racial characteristics, and family were also found to be of importance in this respect. The scheme of inheritance of susceptibility is uncertain, but is apparently of a complex nature. Females that die of toxemia are apparently heterozygous for susceptibility factors, but there is some evidence that males may be homozygous. Homozygous females presumably never reach maturity but die of unknown causes.

The pathogenesis of eclampsia has not been clarified despite long continued study of human cases, but the disorder in the rabbit has enough in common with it to suggest a common or similar mode of origin. Studies of the etiology of the condition in the rabbit are not sufficiently advanced to allow final conclusions, but certain points in connection with the incidence and manifestations are apparently of significance.

The clinical manifestations and many of the pathological changes as in eclampsia are suggestive of an intoxication, but investigation gave no evidence of a toxic substance of extraneous origin and bacteriological examination was negative. Moreover, the occurrence of typical cases of toxemia in pseudopregnant and in postpartum animals eliminates the possibility of a toxic agent arising from the products of conception.

The incidence of cases, however, leaves no doubt that the disorder is associated with the physiological changes incident to pregnancy and the time of occurrence of cases in relation to gestation is apparently of significance. The great majority of cases in pregnant does appeared during the last few days of gestation, cases in pseudopregnant animals occurred in approximately the same interval after mating, and the majority of postpartum cases appeared on the 1st or 2nd day. The behavior observed repeatedly in pseudopregnant does at this period indicates that these animals are subject to the same physiological changes that characterize the terminal stages of pregnancy, and presumably the influences affecting such changes are still active early in the postpartum period.

During the terminal days of pregnancy fetal growth is retarded (9) and the habitus of the mother is in the process of change in preparation for parturition and lactation. The initiation and control of these processes is a function of the endocrine system, particularly of the hypophysis, and it may be assumed that dysfunction of these glands would lead to abnormal stimulation and pathological alteration of the the processes associated with this period. The marked histological alteration of the endocrine system in toxemia of pregnancy in the rabbit is evidence of dysfunction and is worthy of consideration as a possible factor of primary etiological importance.

The suprarenal changes in toxemia are degenerative in character and probably secondary and the thyroid is hypoplastic and inactive. Hypophyseal changes, on the other hand, are productive in nature. There is marked hyperplasia of the cells of the anterior lobe with evidence of irregular secretion. Alteration of the intermediate lobe is also a constant feature of the disease. The cells of this lobe are markedly increased in number and frequently show adenomatous proliferation or invasion of the anterior or posterior lobes. In addition, there is a large amount of interstitial colloid substance, and cysts

containing similar material are common both in the intermediate lobe and in the posterior lobe where Herring bodies are also unusually distinct and numerous.

There have been recent attempts to associate eclampsia in man with hypophyseal dysfunction, especially with an altered or excessive secretion of the posterior lobe (10), but the relationship is not generally accepted and the findings have not been substantiated (11). However, the increased susceptibility to toxemia of pregnancy among rabbits known to transmit abnormalities of an hypophyseal order, together with the microscopic alteration of the gland in the disease, is indicative of an etiological relationship.

The meaning of the histological changes in the intermediate lobe is not clear, but they are indicative of pronounced secretory activity and may be of significance in the disorder. It should be pointed out in this connection that in normal rabbits the microscopic appearance of the cells of this lobe suggests an active secretory function, and it seems improbable that this function is limited to the production of a chromatophorotropic substance.

There is as yet no satisfactory explanation for the occurrence of the present outbreak of toxemia. Cases of the disease have occurred sporadically in the colony for a number of years, but in the period under discussion the incidence assumed epidemic proportions. There has been no evidence, however, to suggest that the disorder was infectious in character. Bacteriological examination has been negative, cases were distributed irregularly throughout the colony and intimate contact between affected and susceptible animals failed to reproduce any symptoms of the disease.

The character of the population has not changed and many animals of the most susceptible genetic groups were present in the colony during periods of low incidence. There is no indication that the course of the outbreak was related to the changed environment of the colony in its new location, but on the other hand, there is considerable evidence of the continued action of an extraordinary set of influences in both locations.

Examination of the records of the colony shows the occurrence of a widespread disturbance in physiological function in the periods preceding and coincident with the outbreak, and it is noteworthy that

previous to the epidemic of rabbit pox in 1932 these same changes were observed in the animals (2). In addition, there was a profound change in organic constitution during the outbreak period, and autopsies of all animals, both those killed for disposal and those dying of toxemia and other causes, have shown an abnormal endocrine situation. There is normally a constant relationship between the weights of the thyroid and the hypophysis, both increasing and decreasing together in different periods of the year (12). Throughout the period, however, this relationship was drastically altered and the hypophysis was extremely large and the thyroid so small that it could be located only with difficulty. It is worthy of note in this connection that in the earlier work of this department when animals were inoculated with syphilis or tumor under similar endocrine conditions, a disease of marked and unusual severity resulted.

It seems probable that there is a causal relationship between the endocrine abnormality and the physiological disturbances observed in the animals, and when considered in association with the hypophyseal alteration characteristic of toxemia, this finding assumes significance as a factor in the genesis of the outbreak. The high susceptibility of animals that transmit and themselves show evidences of hypophyseal abnormalities is also suggestive in this respect, for it may be assumed that in such animals the effect of the changed endocrine relationship would be more pronounced than in the general population.

The nature and mode of action of the influences responsible for the endocrine variation are not known, but the marked effect of various environmental factors on endocrine weights and relationships has been demonstrated (12) and such factors may be the basis of the present imbalance.

SUMMARY AND CONCLUSIONS

No definite conclusions relative to the etiology of toxemia of pregnancy in the rabbit can be drawn from the evidence obtained to date, but certain findings are suggestive and will be investigated further in future studies. These findings suggest that the disorder in the rabbit is a generic variation of eclampsia in man. The incidence, clinical manifestations and pathological lesions indicate that the disorder is of

hypophyseal origin and that the association with pregnancy is due to altered activity of that gland in the terminal stages of gestation.

Hereditary factors related to race and certain constitutional variations were associated with increased susceptibility, but their expression was apparently dependent upon environmental conditions. The association of widespread reproductive disturbances with the outbreak of toxemia suggests a causal relation, and it is assumed that the endocrine imbalance was a primary factor in their genesis and was induced by changed environmental conditions. The general response of the population was manifest in functional disturbances which were of minor severity in normal groups and were expressed as toxemia in inherently susceptible animals.

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THE ABSENCE FROM THE URINE OF PERNICIOUS ANEMIA PATIENTS OF A MOSQUITO GROWTH FACTOR PRESENT IN NORMAL URINE

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The larvae of the mosquito, *Aëdes aegypti*, require for their normal growth and development certain accessory growth substances which they obtain, in nature, from living microorganisms. All attempts to grow the larvae in the absence of living microorganisms proved unsuccessful, until it was found (1) that a medium containing heat-killed yeast and 0.5 per cent Lilly liver extract No. 343 (a partially purified preparation used for the treatment of pernicious anemia) would support normal growth under sterile conditions. The growth factor, designated as factor A, which is present in liver extract, cannot be supplied (2) by the highly purified anti-anemic preparations of Dakin and West (3) and Jacobson and Subbarow (4), showing that factor A and the antipernicious anemia principle are not identical. Factor A does, however, resemble the anti-anemic substance in the following ways. It is abundant in liver and kidney and less so in heart, while body muscle contains very little (2, 5). It can be adsorbed by charcoal and dialyzed through a collodion membrane (2, 6). It is heat-stable in neutral or slightly acid solution, but is destroyed by ashing, by boiling for 1 hour with 0.5 N sulfuric acid, or by exposure at room temperature for 24 hours to 0.5 N sodium hydroxide (2, 3). It is present in small amount in that portion of aqueous liver extract precipitated by 70 per cent alcohol, and in large amount in the precipitate obtained when the 70 per cent alcoholic filtrate is brought to a concentration of 95 per cent alcohol (2, 7).

These resemblances seemed to warrant further investigation. Wakerlin's finding (8) that normal human urine contains a substance

effective against pernicious anemia suggested the assay for mosquito growth factor of urine extracts from normal persons and from patients with aplastic anemia and with pernicious anemia before and after adequate treatment.

Two of us prepared the urine extracts, while the other performed the mosquito growth tests, usually in ignorance of the type of person from whom the extract was obtained.

Methods

1. Preparation of the Urine Extracts.—All the urine passed during a 24 hour period was collected with a few drops of toluene as a preservative and stored in a refrigerator. It was then measured, evaporated under reduced pressure at 55°C. to a volume of about 150 cc., and poured into a volume of 95 per cent alcohol sufficient to give a final concentration of 70 per cent alcohol. The mixture was allowed to stand overnight and was then filtered. The filtrate was concentrated under reduced pressure to about 100 cc. and was poured into enough absolute alcohol to give a concentration of 95 per cent alcohol. After vigorous shaking, a fine flocculent precipitate settled out. This was filtered off and dissolved in 100 cc. of distilled water. The pH was adjusted to 6.0 and the material was autoclaved ½ hour at 120°C.

2. The Mosquito Growth Test.—Significant results can be obtained only if the larvae are reared in the absence of living microorganisms. As in previous work on the nutrition of mosquito larvae (2), 1 to 2 day old eggs of *Aëdes aegypti* were sterilized on the outside and placed in tubes of sterile 0.5 per cent Lilly liver extract No. 343. 4 days later the young larvae were washed in sterile distilled water and inoculated into the experimental tubes. These were prepared by making suitable dilutions of the urine extract with sterile distilled water. Each tube contained a total of 6 cc. of medium and received 0.3 cc. of washed killed yeast suspension. Three larvae were inoculated into each tube. Each urine extract was tried in at least four different dilutions, using at least two tubes for each dilution. Some of the extracts were tested several different times with concordant results. The tubes containing the larvae were held in an incubator at 28° ± 1°C. and were observed daily at first and then every other day for a period of about 20 days, the number of larvae in each instar being noted.

RESULTS

(a) In Urine Extracts.—

It has been shown (2) that in the presence of killed yeast (0.1 cc. per larva) and 0.5 per cent Lilly liver extract No. 343 (or other suitable source of growth factor A) (2) all the larvae reach the third instar on the 3rd day, and nearly all reach the fourth instar on the

TABLE I

The Growth of Aëdes aegypti Larvae in Urine Extracts

Extract*	Concentration (fraction of total volume)	Reaching 3rd stage in 20 days	Average time to reach 3rd stage	Reaching 4th stage	Rating
		per cent	*days*	*per cent*	
C:1	1/3	0	—	0	–
	1/6	0	—	0	
	1/12	0	—	0	
	1/20	17	13	0	
C:2*a*	1/3	Toxic	—	0	+++
	1/6	33	8	0	
	1/12	33	9	0	
	1/20	17	6	0	
C:2*b*	1/6	33	9.5	0	++++
	1/12	33	7	0	
	1/20	66	9	0	
	1/60	17	11	0	
G:1	1/3	17	9	0	+
	1/6	17	6	0	
	1/12	17	6	0	
	1/20	0	—	0	
G:2	1/3	0	—	0	+++++
	1/6	83	7	0	
	1/12	67	5	33	
	1/20	50	8	0	
A:1	1/3	0	—	0	–
	1/6	0	—	0	
	1/12	0	—	0	
	1/20	17	11	0	
A:2	1/6	Toxic	—	0	+
	1/12	17	9	0	
	1/20	17	4	0	
	1/60	0	—	0	
A:3	1/3	83	6	0	++++
	1/6	67	7	0	
	1/12	33	5	0	
	1/20	0	—	0	
	1/60	17	6	0	

* The capital letter refers to the patient, the number to the extract, and the small letter to the trial as described in Table II.

TABLE II

Mosquito Growth Test of Various Urine Extracts

Patient	Description of case	Urine extract	Mosquito growth test
A	Housewife of 61. Paresthesia of extremities, 2 yrs. Gastrointestinal disturbance and anemia, 1 yr. Diminished perception of vibratory sense in the extremities. Erythrocytes 4,720,000; hemoglobin 100%; leukocytes 3,700. No free HCl in gastric juice. Treated twice weekly by intramuscular injection of liver extract (Lederle Laboratories) 1 cc. Full remission effected	1. Before treatment 2. Before treatment 3. After treatment	– + ++++
B	Housewife of 50. Pallor and weakness, 3 yrs. Paresthesia and difficulty in walking, 8 mos. Inadequate therapy had been given. Pallor, lingual atrophy, icteroid sclerae, and absent vibratory sense of the extremities. Erythrocytes 3,900,000; hemoglobin 87%; leukocytes 4,950; mean corpuscular volume 89. No free HCl in the gastric juice. Treated twice weekly with intramuscular injection of 10 cc. liver extract (Eli Lilly and Co.). Complete disappearance of symptoms	1. Before treatment 2. Before treatment 3. Before treatment 4. After treatment	– – + +++
C	Housewife of 39. Weakness and loss of weight, 1 yr. Diagnosis of pernicious anemia. Sore tongue and mouth. Paresthesia of extremities for 2 mos. Erythrocytes 2,080,000; hemoglobin 58%; leukocytes 5,000; mean corpuscular volume 121. No free HCl in gastric juice. Treated every 2 wks. with 10 cc. liver extract (Lilly) intramuscularly. Full remission effected	1. Before treatment 2. After treatment, trial *a* 2. After treatment, trial *b*	– +++ ++++

TABLE II—*Continued*

Patient	Description of case	Urine extract	Mosquito growth test
D	Married clerk of 48. Pallor and weakness, 6 yrs. Diagnosis of pernicious anemia for 5 yrs. with inadequate oral therapy. Paresthesia of extremities, 2 wks. Icteroid sclerae, atrophic glossitis, and diminished vibratory sense. Erythrocytes 1,380,000; hemoglobin 42%; leukocytes 3,100; mean corpuscular volume 140. No free HCl in gastric juice. Treated twice weekly with 10 cc. liver extract (Lilly) intramuscularly. Full remission effected	1. Before treatment, trial *a*	+
		1. Before treatment, trial *b*	++
		2. After treatment	+++++
E	Italian-born male cigar worker of 54. Weakness and loss of weight, 2½ yrs. Oral liver extract, 1 yr. Paresthesia and loss of coordination, 1 yr. Marked loss of perception of vibration and position. Erythrocytes 2,490,000; hemoglobin 76%; leukocytes 4,400; mean corpuscular volume 133. No free HCl in gastric juice. Treated twice weekly with concentrated liver extract (Lederle) intramuscularly. Full remission effected	1. Before treatment	−
		2. Before treatment	−
		3. After treatment	++
		4. After treatment	+
F	Single seamstress of 55. Pallor and weakness, 5 yrs. Diagnosis of pernicious anemia for 4 yrs. with inadequate treatment. Paresthesia for 4 mos. Icteroid sclerae, atrophic glossitis, and diminished vibratory sense. Erythrocytes 1,400,000; hemoglobin 50%; leukocytes 4,350; mean corpuscular volume 149. No free HCl in gastric juice. Treated twice weekly with 10 cc. liver extract (Lilly) intramuscularly. Full remission effected	1. Before treatment	+
		2. After treatment	++

TABLE II—*Continued*

Patient	Description of case	Urine extract	Mosquito growth test
G	Housewife of 65. Stomatitis, pallor, weakness, 8 mos. Paresthesia, 2 mos. 30 lbs. loss of weight. Atrophic glossitis, icteroid sclerae, and diminished vibratory sense of the extremities. Erythrocytes 710,000; hemoglobin 22%; leukocytes 1,950; mean corpuscular volume 129. No free HCl in gastric juice. Treated twice weekly with 10 cc. liver extract (Lilly) intramuscularly. Full remission effected	1. Before treatment 2. After treatment	+ +++++
H	Married stationary engineer of 64. Pallor and weakness, 2 yrs. Diagnosis of pernicious anemia with inadequate therapy. Icteroid sclerae. Vibratory sense absent in lower extremities. Erythrocytes 1,400,000; hemoglobin 40%; leukocytes 2,700; mean corpuscular volume 122. No free HCl in gastric juice. Treated twice weekly with 10 cc. liver extract (Lilly) injected intramuscularly. Full remission effected	1. Before treatment 2. After treatment	++ ++++
I	Married unemployed male of 40 with stomatitis, weakness, and pallor for 7 yrs. Inadequate oral treatment with liver extract. Icteroid sclerae and very slightly diminished vibratory sense in the extremities. Erythrocytes 2,600,000; hemoglobin 74%; leukocytes 6,650; mean corpuscular volume 112. No free HCl in gastric juice. Treated by ultraviolet light for 2 wks. with moderate improvement of the blood. Treated twice weekly with 10 cc. liver extract (Lilly) injected intramuscularly. Full remission effected	1. Before treatment 2. After ultraviolet treatment 3. After liver extract treatment	– ++ ++++

TABLE II—*Concluded*

Patient	Description of case	Urine extract	Mosquito growth test
J	Cancer of intestine	1	+
K	Ulcerative colitis	1	+
L	Aplastic anemia	1	+++
M	" "	1	+++++
N	" "	1	++++
O	" "	1	++++
P	" "	1	+++
Q	Probable aplastic anemia	1	++
R	" " "	1	++++
S	Leukemia	1	++
T	"	1	++++
U	Normal	1	+++
V	"	1	+++++
W	"	1	++++
	"	2	+++

4th day and emerge as adult mosquitoes on the 9th day. With the same amount of killed yeast in distilled water (or in various other media not containing factor A) the larvae never get beyond the second instar, and they eventually die in this stage.

Preliminary trials with normal urine extract showed that while many of the larvae reached the third instar, only a few reached the fourth and none emerged as adults. Thus normal urine extract either does not contain enough factor A to bring about normal growth, or else contains only some of the substances which are responsible for the factor A activity. Concentrations of urine extract higher than 40 per cent by volume were generally toxic, all the larvae being dead 1 day after inoculation. In the middle range of concentrations, depending on the urine extract used, the larvae either survived for a long time in the second instar or reached the third instar and then survived at this stage.

Accordingly, the percentage of larvae reaching the third instar within 20 days was taken as the chief criterion of growth. The other criteria were the average time to reach the third instar and the percentage reaching the fourth instar (very small even in the most favorable cases). On the basis of these criteria the urine extracts were rated with respect to their growth factor content as −, +, ++,

etc. Table I gives some of the actual data and illustrates the method of rating.

The results with the various urine extracts tested and rated in this manner are given in Table II.

TABLE III

Mosquito Growth Test of Urine Extracts in the Presence of the Calcium-Filtrate Fraction†*

Medium		$N \times \frac{1}{T}$
Calcium-filtrate fraction only		5.1
Calcium-filtrate + flavine-purine complex (100 cc. of solution has material from 50 gm. liver)		23.1
Calcium-filtrate + urine extract W:2‡	1/6	16.0
	1/12	15.0
	1/24	12.2
" " " " C:2	1/6	13.6
	1/12	15.8
	1/20	10.3
" " " " D:1	1/6	7.4
	1/12	11.5
	1/20	7.1
" " " " D:2	1/12	17.1
	1/20	15.4
	1/60	10.1
" " " " A:2	1/12	9.2
	1/20	0
	1/60	0

* Concentration of urine extract expressed as fraction of total volume.

† Concentration of calcium-filtrate fraction always such that 100 cc. of solution contains the material derived from 50 gm. of liver.

‡ See Table II for description.

(*b*) *In Urine Extracts Supplemented with Certain Liver Fractions.*—Work, as yet unpublished, has shown that the mosquito growth factor A consists of at least two components.[1] One of these was iso-

[1] The work was done in collaboration with Dr. Y. Subbarow of The Harvard

lated as a flavine-purine complex (1.2 per cent flavine-phosphate). The other was present in a fraction (designated as calcium-filtrate fraction) derived from the material obtained from liver extract by adsorption on charcoal and elution with alcohol (6). In the presence of killed yeast, neither of these two fractions alone supported normal growth of the larvae. But both together, in a concentration such that 100 cc. of solution contained that amount of each which was derived from 50 gm. of liver, gave entirely normal growth and metamorphosis. The method employed for the quantitative assay of factor A has been previously described (2). A growth index is obtained as a number, $N \times \frac{1}{T}$, which is determined by the percentage of larvae reaching the fourth instar in 10 days and the average time required to reach the fourth instar. When growth proceeds at an optimum rate, the value of $N \times \frac{1}{T}$ is $100 \times \frac{1}{4}$ or 25. Five of the urine extracts were tested in the presence, first, of an optimum concentration of flavine-purine complex and second, of an optimum concentration of calcium-filtrate factor. Growth in all the urine extracts plus flavine-purine complex was the same as in the urine extract alone, so that $N \times \frac{1}{T}$ equalled zero. But normal urine extract plus calcium-filtrate fraction gave growth almost as good as in flavine-purine complex plus calcium-filtrate fraction. These results are shown in Table III. It is worth noting that in this test, as well as in the test shown in Table II, extracts C:2, rated as +++, and D:2, rated as +++++, gave growth as good as did the normal extract W:2, rated as +++, while the growth obtained with D:1 and A:2, both rated as +, was markedly less.

DISCUSSION

The data of Table II demonstrate that normal urine, as well as urine from persons with aplastic anemia or leukemia, contains a substance which, under the described conditions, will enable many *Aëdes aegypti* larvae to reach the third instar, and a few to reach the fourth instar. In the urine of nine pernicious anemia patients this substance was absent or present in much smaller amount. Following a full remission of symptoms produced by liver extract therapy the

urine from all of these patients showed an increased amount of the mosquito growth factor. In the urine of seven of the nine patients the amount of this substance was greatly increased, reaching or slightly exceeding that present in normal urine. Interestingly enough, the urines from a patient with cancer of the intestine and from one with ulcerative colitis also showed a low content of growth factor.

The data of Table III give some indication as to the nature of this growth substance. Normal urine extract, or extract from the urine of adequately treated pernicious anemia patients, can replace the flavine-purine complex which is necessary for the growth of the mosquito larvae. Extract from pernicious anemia patients who show symptoms cannot replace the flavine-purine complex, giving growth but little better than that obtained with the calcium-filtrate fraction alone. Neither normal nor pernicious anemia urine extracts can replace the calcium-filtrate fraction. Hence we can tentatively conclude that the flavine-purine complex, or some material endowed with its potentialities for mosquito development, is excreted in much smaller amount by pernicious anemia patients showing symptoms than by normal individuals, persons with aplastic anemia, or adequately treated pernicious anemia patients. Normal urine extract, since it does enable a few larvae to reach the fourth instar, must also contain very small amounts of substances having effects like those of the calcium-filtrate fraction. The data thus far obtained give no information concerning the presence or absence of these substances in pernicious anemia urine extracts.

Several workers have shown that appreciable amounts of free flavine are excreted in normal human urine (9–13). No work has yet been reported on the flavine excretion of pernicious anemia patients. There is sufficient evidence that riboflavine is neither the anti-anemic factor (14), the "extrinsic factor" (15) or the pellagra preventive factor (16, 17). Nevertheless, it is still possible that there is, in pernicious anemia, an upset in the flavine metabolism. In this connection, the work of Laszt and Verzár (18) on chronic iodoacetate poisoning of rats is of especial interest. These investigators found that rats fed on a complete diet containing suitable amounts of iodoacetate failed to grow, and developed steatorrhea, osteoporosis,

skin symptoms, a decided anemia, and great hypertrophy of the suprarenals. All the effects could be completely counteracted if the rats were fed flavine-phosphate, but not if they were fed lactoflavine, indicating that the poisoning interfered with the phosphorylation of lactoflavine, a reaction necessary in the formation of yellow enzyme (19).

Miller and Rhoads (20) have shown that the livers of swine fed a modified Goldberger diet are deficient in antipernicious anemia substance. They have also found (21) that guinea pigs kept on this diet lose weight rapidly and die in 2 to 3 weeks unless the diet is supplemented with adequate amounts of liver extract or vegex, when the animals remain in normal health. An extract from the liver of a swine on this diet, and three extracts from the livers of groups of guinea pigs on this diet, were found to contain very much less mosquito growth factor A than normal swine and guinea pig liver extracts respectively. Unfortunately, these deficient extracts were not tested in such a manner as to determine whether they were lacking in flavine-purine complex or in calcium-filtrate fraction or in both components of factor A.

SUMMARY

Extracts prepared from the urine of normal persons or patients with aplastic anemia or leukemia contain a substance, possibly flavine or a flavine compound, which under suitable conditions of test enhances the growth of larvae of the mosquito, *Aëdes aegypti*. This substance is lacking, or is present in much smaller amount, in extracts from the urine of pernicious anemia patients showing symptoms of the disease. Extracts from the urine of the same patients after adequate treatment contain as much of the substance as normal urine extracts.

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STUDIES ON STRONGYLOIDES

II. Homogonic and Heterogonic Progeny of the Single, Homogonically Derived S. ratti Parasite[1]

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The establishment of fertile *Strongyloides ratti* by means of a single larva of homogonic development, and its continuation by serial passage through additional generations in the rat host has already been reported (Graham, 1935; 1936). The methods employed in this work have afforded observations on the character of the offspring of this parasite under the simplest and, presumably, most ideal conditions in which a nematode parasite of mammals can live; namely, one worm dwelling without competition in its normal environment. There is thus a minimum of interference by the host through the agency of immune reactions, etc.

The inference seems justified that the analysis which such a study permits furnishes a true picture of the complete, biological potentialities of a single *Strongyloides ratti* derived from a homogonic larva, as to number and type of progeny thus produced. Within these limits there is new orientation as to what facts should be taken into consideration in explaining the appearance of offspring differentiated as to homogonic and heterogonic type.

The present study is based upon 5,118 positive, daily progeny yields as determined by the examination of cultures made from 24-hour fecal collections from 116 rats each harboring a parasite ranging in age from 1 to 55 weeks.

[1] The writer is indebted to Dr. Norman R. Stoll for valuable suggestions and criticism during the course of these studies.

MATERIALS AND METHODS

The techniques employed in these studies have been presented in detail in a previous paper (Graham, 1936). For over a year incubation of the fecal cultures has been carried out in an electric incubator operating at 27.5 to 28.5° C. In general, cultures have been incubated for 3 days but, during a period (June–July) when the room temperature rose above that at which the incubator was set to operate, they were isolated after 2 days' incubation. On a few occasions cultures were isolated after an incubation period of only 1 day.

Attention is called to the homogeneity of the host material which has been employed. The yellow-hooded rats were a highly inbred stock when they were first used for single larva infections of *S. ratti* over 2 years ago. At that time they had been bred brother to sister for over twenty generations, the degree of inbreeding prior to that being unknown to the writer. The inbreeding has been continued up to the present time.

The strain of *S. ratti* has been continued without change. The original stock of the parasite has been maintained and the pure-line strain established by single larva infections has similarly been continued.

RESULTS

Continuation of Serial Generations of S. ratti Established by Single Larva Infections

At the present time the initial, single larva strain has been passed serially through 34 parasitic generations. The detailed data concerning the first fourteen generations have been published (Graham, 1936) with tables and figures indicating the serial parentage, etc.

For the establishment of the last twenty serial generations of the initial, single larva strain, a total of 259 rats were exposed to a single larva each. Of these, 46 (18 per cent) became infected as indicated by the appearance of progeny in fecal cultures secured from these animals.

In addition to the continuation of the initial strain, three new strains of *S. ratti* were isolated from laboratory culture stock and passed through the first parasitic generation by means of single larva

infections. From one of these new strains, a second generation was established before it was discontinued. For these new strains, 29 rats were exposed to a single larva each, and sixteen became infected.

Thus, a total of 469 rats have been exposed to a single, direct development larva each and 120 (25.6 per cent) have become infected. Considering, for the moment, only those animals utilized in establishing the initial, single larva strain and successfully carrying it through 34 parasitic generations, it should be pointed out that, for the first fourteen generations, the exposure of 185 rats to a single larva each yielded 58 (31 per cent) demonstrably infected animals. (At the time attempts to establish generation XVI were being made, homogonic larvae from a surviving infection of generation X were used on four rats unsuccessfully. These four negatives changed the totals from 181, 58, and 32 per cent, as given by Graham (1936, p. 86), to 185, 58, and 31 per cent as noted.) For the last twenty generations the percentage of infection was 18 per cent. This reduction of the infection rate should not be interpreted as a gradual loss of infectivity due to exhaustion following repeated passage by means of a single homogonic larva. Of the 259 rats used to carry the strain through the last twenty serial generations, 112 were utilized for generations XV to XVIII inclusive and yielded only nine positive infections; whereas, of the 143 rats used for generations XIX to XXXIII inclusive, 37 (26 per cent) of the rats exposed to a single larva became infected. Of this latter group, the 37 rats used for generations XXX to XXXIII inclusive yielded thirteen, about one-third, positives. It is clear that there is no reason for belief in a progressive decline in infectivity of the strain at the present time.

Type of Progeny Produced by Single S. ratti As Related to Age of Parasite

1. *General considerations.* The basic data for this analysis are the progeny counted from Baermann isolations of cultures made from 24-hour fecal collections from rats each infected with a single, homogonically derived *S. ratti*. These counts were made only from isolations of cultures of fecal pellets which were incubated as collected, neither crushed nor mixed with charcoal. Discarded were 808 counts made from cultures collected from the first 22 rats which were infected with a single parasite, serial generations I to IV inclusive. Counts

made from fecal cultures collected from eighteen of these 22 rats were admissible for this analysis from the later stages of the infections. A total of 116 single, homogonic *S. ratti* infections are involved.

In regard to discarding the 808 counts, it was reported earlier (Graham, 1936) that several methods of culturing the feces had been used; among them, mixing with either powdered or granular animal charcoal, crushing and mixing the pellets without charcoal, and culturing the pellets directly as recovered from the collection pans without crushing or mixing. The statement (p. 75) that "Little, if any, difference was discernible in larval yields by the different methods" was made on the basis of gross inspection of raw data and intended only to justify the adoption of a technical short-cut; i.e., culturing the fecal pellets without mixing or crushing. A comparison of culture yields from eighteen single *S. ratti* infected animals where two or more contiguous daily counts were available, one from an unmixed culture and the other from a culture mixed with, or without, charcoal, was made. From 159 "mixed" cultures a total of 490 offspring were enumerated, or 3.1 per culture. From 141 "unmixed" cultures, a total of 1,080 offspring were obtained, or 7.7 per culture. If it can be fairly assumed that samples of this size; i.e., 300 cultures, are adequate to disperse variations in progeny yield due to normal fluctuation, then it follows that the mixing of cultures, with or without charcoal, results in the loss of over one-half of the progeny.

A total of 5,118 daily cultures with one or more offspring, secured from 116 single worm infections of *S. ratti*, provided a basis for the classification of homogonic and heterogonic progeny of *S. ratti* insofar as the production of these two types of progeny was related to the age of the single, homogonically derived parasite. That such aging of the *Strongyloides* parasite (as well as of the host) might be important in determining the mode of larval development has been variously suggested in the literature, though no definitive data to support the speculation have been adduced. Consideration of this possibility has constituted a partial objective of the present investigations concerning the biology of *S. ratti*.

Although the rats used over a period of more than 2 years were of different parentage, age and sex, and the homogonic larvae of different parental history and degrees of inbreeding, the material in general

possessed unusual homogeneity. Of the 116 single *S. ratti* infections which contributed data for this study, 92 were derived serially from a *single* homogonic larva. The remaining 24 single larva infections were obtained with homogonic larvae derived from the stock infection of *S. ratti*, which has been carried mainly, if not exclusively, by homogonic passage since it was first secured from wild rats (Graham, 1936, p. 74). For the purpose of analysis it has been assumed that this material is comparable throughout. Slight differences existing between the data from these two groups of infections are considered of minor importance and discussed later.

In many instances the progeny counts were made intermittently, although frequent long periods of continuous daily counts were also recorded. In comparatively few cases were progeny yields from the single parasites obtained without break from the onset of patency until reproduction ceased.

2. *The frequency with which progeny of heterogonic development appeared in cultures.* The individual daily progeny yields have been divided for analysis into groups according to the age in weeks of the parasite from which they originated. This method provided groups containing 100 or more positive cultures for 22 of the 55 weeks of the age range obtained.

The cultures contained progeny, distributed by type, in three combinations: (1) those with only heterogonic adults, (2) those with only homogonic larvae, and (3) those with both homogonic larvae and heterogonic adults. Using indirect and direct as synonyms respectively for offspring of heterogonic and homogonic development, classification of "total indirect," "pure indirect," "total direct," "pure direct," and "mixed direct-indirect" cultures was made for each week of parasitic age. In a small number of instances, where larval progeny of heterogonic adults as well as these adults themselves were obviously mixed with larvae of direct development, no attempt was made to distinguish between the larvae involved. The adults of indirect development were enumerated and the cultures excluded from all analyses except the present one where classification as a mixed culture was assuredly correct.

In figure 1, the results are set forth graphically. The overwhelming preponderance of cultures, over 98 per cent, which contained larvae of

direct development, is expressed in the "total direct" curve *A*. This is the composite of curves *B* and *D*. The percentage which contained only adults of heterogonic development (= pure indirect) is not separately shown in figure 1 because it is exemplified in the difference between curves *C* and *D*. It is also the obverse of curve *A*. Exclu-

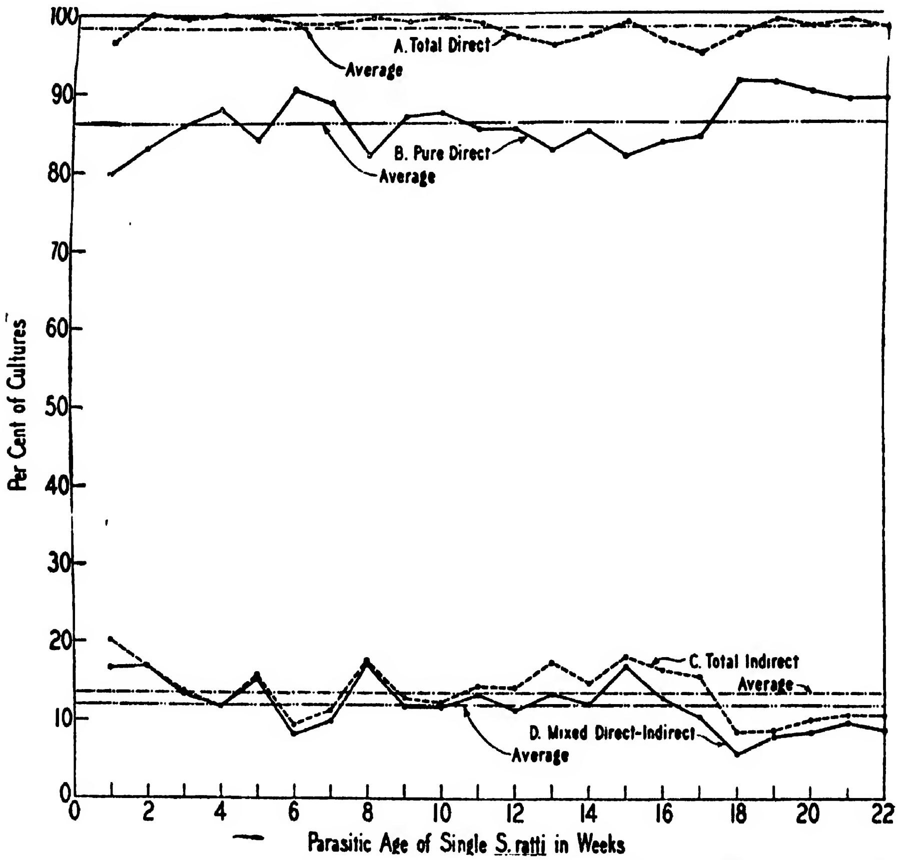

FIG. 1. Classification by presence or absence of offspring of direct and indirect development in 4,209 cultures (basis 24-hour fecal collections) from 116 single, homogonically derived *S. ratti* parasites in the rat host.*

sive of the first week of parasitism, less than 1.5 per cent were "pure indirect" until after week XI.

Of particular interest in figure 1 is curve *C* of "total indirect" cultures. As indicated by the centering line, which represents the average of the 22 weeks plotted, nearly 14 per cent of the cultures

* See also table 1.

showed progeny of indirect development. The significance of the terminal drop in curve *C* (which is, of necessity, reflected in the other curves, beyond week XVII) will be discussed later. The obverse of curve *C* is expressed in that of "pure direct" cultures (*B*: 86 per cent).

Notable is the relative condition prevailing in the weeks beyond those indicated in figure 1 which, though covering 60 per cent of the observed age range, contained only 18 per cent of the available positive cultures. None of these weeks (XXIII to LV) contained 100 positive

TABLE 1

*Classification by Presence or Absence of Offspring of Direct and Indirect Development in 5,118 Cultures (Basis 24-Hour Fecal Collections) from 116 Single, Homogonically Derived S. ratti Parasites in the Rat Host, with Special Reference to Weeks XXIII to LV of Parasite Age**

Age of parasite in weeks	Positive cultures	Percentage of cultures showing			
		Total direct	Pure direct	Total indirect	Mixed direct-indirect
XXIII to XXIV	144	97	87	13	10
XXV to XXVI	125	99	91	9	8
XXVII to XXVIII	104	98	93	7	5
XXIX to XXX	100	92	87	13	5
XXXI to XXXIII	103	98	87	13	11
XXXIV to XXXVII	107	99	93	7	6
XXXVIII to XLIII	104	99	95	5	4
XLIV to LV	122	89	79	21	10
I to XXII	4,209	98.3†	86.3†	13.7†	12.0†
I to LV	5,118	98.2‡	86.5‡	13.5‡	11.7‡

* See also figure 1.

† Average by weeks.

‡ Whole series, not weekly basis.

cultures, so they have been grouped consecutively to obtain at least this number as shown. In table 1 it will be seen that, although the cultures containing progeny of indirect development fell rather low at several points, these data in no way suggest that the ability of single *S. ratti* to produce offspring of heterogonic development has been impaired as a result of age. In fact, it is apparent that single, homogonically derived *S. ratti* produce progeny of indirect development throughout their entire reproductive life at approximately the same

frequency. It is to be noted that 21 per cent of the cultures obtained during weeks XLIV to LV contained offspring of heterogonic development, the same percentage that was observed for the first week of the infections.

The average of the entire series (22 weeks plus eight groups covering 33 weeks) for "total indirect" cultures is 12.9 per cent, a value which is very close to that obtained for the first 22 weeks (13.7 per cent), the average of the last 33 weeks (eight groups) being 10.8 per cent.

At the bottom of figure 1, curve *D* is shown expressing the percentage of cultures with both larvae of direct development and adults of heterogonic development (= "mixed direct-indirect"). As indicated by the centering line, 12 per cent of all cultures for the first 22 weeks were in this category. It will be recognized that curve *C* ("total indirect") is the composite of curve *D* (= "mixed direct-indirect") and the obverse of "total direct" curve *A*; i.e., "pure indirect."

It is obvious that the predominant mode of larval development was the direct type, although an appreciable number of cultures were obtained containing indirect forms. The constancy of these relationships suggests that neither age of parasite nor age of host is the significant factor in explaining the indirect mode of development occurring in single *S. ratti*.

3. *The percentages of all progeny from single* S. ratti *which were adults of indirect development.* As has been pointed out above, offspring of heterogonic development are produced throughout the entire reproductive life of the parasites at a fairly regular rate as far as the age of the parasites is concerned. A study of the numbers of progeny and the percentage that were of direct and indirect type produced further information of interest.

In figure 2 are given the average daily *numbers* of progeny of indirect and direct development (as contrasted merely with their presence or absence in the cultures; cf. fig. 1) for each week of age from I to XXII. Beyond week XXII, where there were fewer than 100 cultures available per week, grouping was employed to provide at least that number for the establishment of points on the curve. Those earlier mentioned cultures, from which an exact count of larvae of direct development could not be made because of the presence of larval

progeny from the adults of heterogonic development, were omitted from these data. It is apparent from this figure that, while adults of indirect development were not numerous, they were produced regularly throughout the entire range of patent infection. Direct larval offspring were most numerous during the first 2 months of infection, then a low level of their production was reached which persisted for many weeks and declined very gradually. It is this declining production of larvae of homogonic development which, obviously, results in a relative increase in the percentage of the indirect forms.

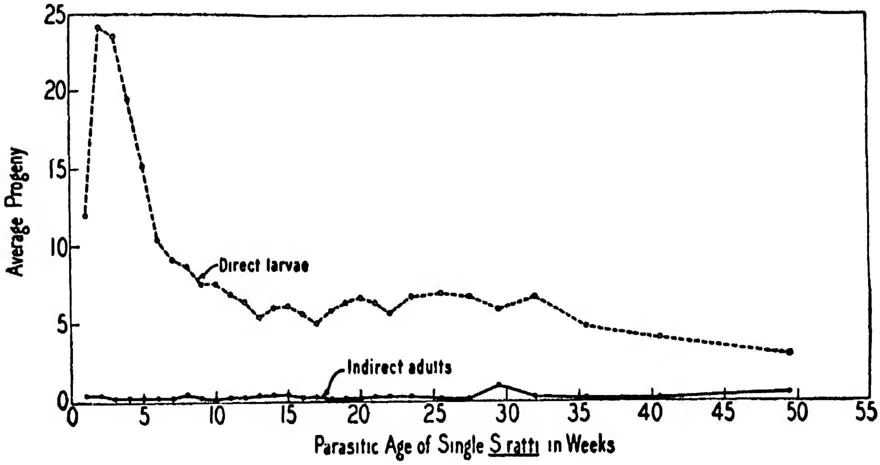

FIG. 2. Average numbers of homogonic larvae and heterogonic adults in the progeny secured from 116 single *S. ratti* parasites, as observed in cultures made from 24-hour fecal collections from the rat host. In each of the first 22 weeks, 100 or more cultures contained one or more progeny. Beyond this range, the data are grouped to provide 100 or more such cultures.

Another expression of this is given in figure 3. For establishing curve *E*, the total progeny produced were grouped by weeks in units of approximately 10,000 progeny and the percentage of heterogonic progeny determined. Thus five points were defined: weeks I and II, containing 13,464 progeny; weeks III and IV, 11,796; weeks V to VIII, 10,592; weeks IX to XVI, 9,216; and weeks XVII to LV, 9,799. In all, 54,867 progeny were enumerated and classified from the 5,118 daily cultures. The curve thus described is, in effect, the obverse in type of that depicting the average yield of progeny of direct development in figure 2.

A cumulative curve, *F*, is also presented in figure 3, successive points representing the percentage of progeny of indirect development in weeks I to IV, I to VIII, I to XVI, and I to LV. Over the entire 55-week range this was 2.3 per cent of the total (54,867) progeny. The progressive increase in the percentage of progeny of indirect development is due, not to their more frequent production, but rather to the decreasing production of larvae of direct development.

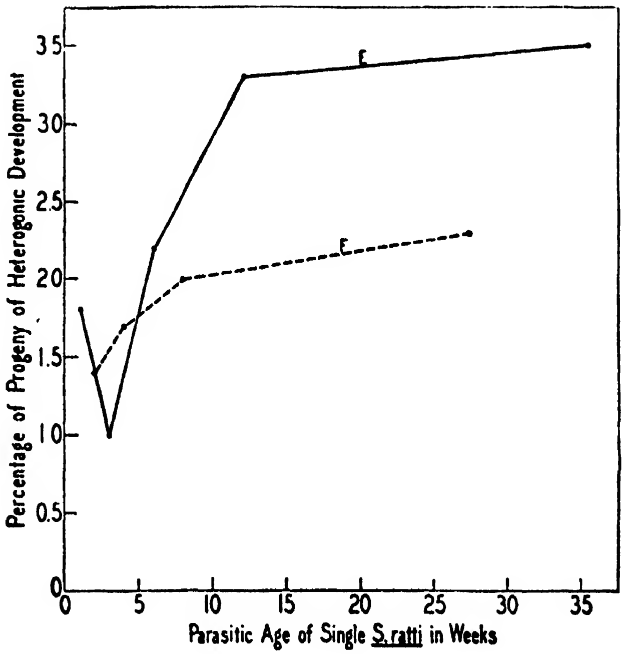

FIG. 3. The percentage of adults of indirect development among 54,867 progeny. The points of curve *E* represent such percentages for consecutive units of approximately 10,000 progeny each, covering weeks I to II, III to IV, V to VIII, IX to XVI, and XVII to LV respectively. Curve *F* is a cumulative curve derived from curve *E*, the four points indicating percentages of offspring of indirect development as of weeks I to IV, I to VIII, I to XVI, and I to LV respectively.

In the first 22 weeks, 2.1 per cent of all progeny were free-living adults; in the remaining 33 weeks, which contained only 10 per cent of the total offspring enumerated in the entire series, 4.2 per cent were observed. While it is true that, in the later weeks of this age range, both the number of positive cultures and the actual number of offspring are decidedly small in comparison with the earlier weeks, never-

theless they afford no reason for believing that the as yet unknown factor, or factors, governing the mode of larval development have reached a biological turning point.

Considering the whole study, it is clear that the curves of classified culture yields shown in figure 1 cannot be considered as essentially different from a straight line; i.e., the fluctuations are not of a significant order. This interpretation is adequately supported by the data introduced in conjunction with figures 2 and 3 concerning the average yield and the percentage of the two types among the total progeny. Thus, it appears that neither the age of the parasite nor the age of the host makes any important contribution in determining the mode of development which the progeny of single, homogonically derived *S. ratti* pursue.

DISCUSSION

The foregoing data provide evidence of a kind that has not heretofore been available in considering the foremost *Strongyloides* problem; namely, the factors responsible for the bimodal type of larval development. It clearly defines the biological reaction of one species with regard to one important factor; i.e., the kind of progeny produced by a single, homogonically derived *S. ratti* as related to the age of both host and parasite. Progeny of indirect development occurred in 13.5 per cent of the cultures but only slightly more than 2 per cent of all progeny were adults of the free-living generation. This was remarkably uniform over an age range of 55 weeks. Considered on either the basis of the percentage of cultures which contained progeny of indirect development or the frequency of these heterogonic adults in the total progeny, the data indicate that these heterogonic offspring appeared independently of the age of the parasite. They are susceptible to no other interpretation.

It is obvious, conversely, that the progeny shed by single *S. ratti* are predominantly larvae of direct development. A similar condition has been observed in this laboratory in mass infections of the parasite. It is interesting to observe that, whereas only slightly over 2 per cent of the progeny from the 116 single *S. ratti* studied in the present case were indirect forms, Sandground (1926) gave protocols of mass infections of *S. ratti*, established with selected, homogonic larvae, in

which 7.3 per cent of the progeny enumerated were free-living adults. The longest period of examination that he recorded was 22 weeks. In two infections of this range six examinations were made in one case and nine in the other.

Sandground (1926) also concluded that it was not "possible to make any correlation between the age of the parasites, and the mode of development of their larval offspring." The present findings provide striking confirmation on this point. They are based on a much larger series of individual infections than he employed and the progeny yields, in addition to being much more numerous than in his study, more nearly cover the entire reproductive life of the parasites involved. Finally, in the present study, where each infection was established by means of a single larva, there is no necessity for considering the possibility that a hypothetical male parasite has played a part in producing the observed results (Graham, 1936).

Concerning the two classical concepts of the causal factors; i.e., chromosomal vs. environmental influence, responsible for the bimodal type of larval development which characterizes many, if not all, species of the genus *Strongyloides*, the available data do not allow a clear choice to be made except for the species involved in this study, *S. ratti*. The present evidence indicates that environment, as represented in the fecal cultures during the incubation period, is not the determinative factor. In the first 8 weeks of the infections, which reference to figure 2 will show covered the period of maximum fecundity, and during which time 65 per cent of the nearly 55,000 progeny were procured, the *percentage* of the total weekly offspring that were adults of indirect development was depressed only as the yield of larvae of direct development increased. Then, as the yield of larvae of homogonic development decreased, the *percentage* of progeny of heterogonic development became increasingly greater. The numbers of progeny of indirect development during this first 8-week period were 3.0, 1.7, 0.9, 1.0, 1.5, 2.0, 2.4, and 3.9 per cent of the total progeny in each week, respectively. In view of the large number of organisms observed, it seems obvious that the mode of larval development which the progeny of single *S. ratti* pursued was not primarily determined in the environment of the fecal cultures.

Inasmuch as the trough-like character of the curve depicted by the

above-mentioned percentage yields is evidently not the type necessary to establish the fecal culture as a prime environmental influence in determining the mode of larval development of *S. ratti*, the weight of the present evidence forces tentative acceptance of chromosomal influence as an alternative. This is in harmony with Sandground's (1926) suggestion that it is "determined by the inherent constitution of the egg from which the larva develops." It is possible that the small, though regular, numbers of progeny of heterogonic development appear because some mechanism *within the parasite* operates at a potentially fixed level and more or less irrespective of the total number of ova being shed. The operative factors which produce this constitutional difference in the ova, are as yet, unknown.

In this connection the earlier mentioned differences between the data obtained from the 92 pure-line infections and the 24 extra infections may appropriately be considered. The single serial line which represented 79 per cent of the 116 infections furnished 81 per cent of the 4935 culture days under review. For the first 45 weeks studied (only the pure serial line extended into the 45 to 55 week period) the larger group produced indirect progeny on 11.9 per cent and the smaller extra group on 21 per cent of the culture days, while the figure for both groups for the period as given was 13.6 per cent. In the larger group 1.9 per cent of the total progeny were of indirect development, in the extra group 3.5 per cent.

This would appear to indicate, which evidence from the study of additional single larva infections of *S. ratti* of homogonic origin suggests, that all homogonic larvae are not constitutionally equivalent. Whatever variation the single larva infections under consideration may show between themselves appears, however, to be of a wholly different magnitude from single larva infections established with heterogonically derived forms. The predominance of one serial line in the homogonic series under study in this article thus appears to have added value.

Whatever the ramifications of the problem, the type of offspring produced must eventually be explained, in *S. ratti*, on a basis adaptable to a single worm, even though the parasite has been selected for homogonic development for several generations by means of single larva transfers. Either syngonism (Sandground, 1926) or partheno-

genesis in this species can logically be accommodated by this single parasitism. On the other hand, bisexualism in the parasitic phase of *S. ratti* has not yet been observed, nor are the facts concerning the bionomics of the species made clearer by its consideration. Both male and female adults of the free-living generation, as well as direct development larvae, were obtained among the progeny of single females of homogonic origin. A similar observation was made by Beach (1936) with isolated parasites of *Strongyloides simiae* studied in vitro, the parasites presumably being of heterogonic origin. Chu (1936), working with the related genus *Rhabdias*, observed that "both types of development were found to occur among the eggs from a single parasitic worm" of *R. fuscovenosa catanensis*. His search for a parasitic male of this snake parasite was fruitless and he concluded "that a functional male would be superfluous." A similar conclusion was reached by Graham (1936) concerning a hypothetical male of *Strongyloides ratti*.

Insofar as the central problem of *Strongyloides* biology is considered to be an explanation of the mode of larval development, the present study provides a more adequate, factual basis upon which to approach it for *S. ratti*.

SUMMARY

1. The types of daily progeny from 116 single *S. ratti* over a parasite age range of 55 weeks, were studied on the basis of cultures from 24-hour collections of feces from the rat host. About 14 per cent of the cultures contained adults of indirect development. The frequency with which cultures containing progeny of indirect development appeared did not vary significantly as the age of the parasite increased.

2. The numbers of adults of indirect development and larvae of direct development were determined for the first 22 weeks of infection. Beyond this point, weeks were grouped consecutively to give a minimum of 100 cultures for similar determinations. Whereas the number of progeny of indirect development remained remarkably stable over the entire age range, the progeny of direct development reached a maximum in week II, declined abruptly for about 6 weeks, and then declined further at a very slow rate.

3. The total progeny, grouped in five blocks of approximately 10,000 each, and covering weeks I to II, III to IV, V to VIII, IX to XVI, and XVII to LV, respectively, were employed to determine the percentage yields of offspring of indirect development. The small variations thus demonstrated, which ranged from 1.0 to 3.5 per cent, were shown to depend on the variations in the numbers of larvae of direct development and not on the numbers of progeny of indirect development. Adults of heterogonic development constituted only slightly more than 2 per cent of the total progeny.

4. Neither the age of the parasite nor the age of the host had any significant rôle in determining the mode of development which the progeny of a single, homogonically derived *S. ratti* pursued.

5. The failure of the progeny of indirect development to increase and decrease in parallel with the larvae of direct development during the period of maximum fecundity in weeks I to VIII is interpreted as evidence that the mode of development pursued by the progeny of single, homogonically derived *S. ratti* was not determined in the environment encountered by the young larvae in the fecal cultures during incubation. Presumably the mode of development is already determined at the time of oviposition.

6. The isolated, pure-line strain of the parasite has been passed serially through 34 parasitic generations. Of 469 rats exposed to a single, homogonic larva each, 120 (25 per cent) became demonstrably infected. No evidence of decreased infectivity was detected as a result of this prolonged restraint of heterogony.

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TEST OF A THEORY ON THE ORIGIN OF BACTERIOPHAGE[1]

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Two theories concerning the nature of bacteriophage have been widely championed: first, that 'phage is a living virus which invades the bacterial cell and propagates within it; and second, that 'phage is a non-living substance derived from bacterial metabolism and having the capacity to increase in amount when brought in contact with susceptible microörganisms. According to Krueger (1936), "hypotheses embodying either of these concepts together with varying subsidiary corollaries have been advanced in considerable numbers and the arguments centering around them constitute a formidable fraction of phage literature."

Although the known facts, as Krueger says, seem to favor a bacterial origin for 'phage, another theory has not been sufficiently tested experimentally. It may be that 'phage originates within the animal harboring certain bacteria and is produced as a reaction or as an autocatalytic antibody against the microörganisms. To test this notion one would have to rear an animal under perfectly sterile conditions and find it consistently free of 'phage. Then it would have to be infected with a non-lysogenic but lytically susceptible bacterium for varying periods of time, after which the animal would again be tested for 'phage. If 'phage then appeared, it would mean that the tissues of the animal had responded by forming 'phage or, at least, had participated in the reaction.

The housefly (*Musca domestica* L.) was the animal chosen for this work because it seemed likely that this species could be reared under

[1] The writer wishes to thank Mr. N. A. Coria for valuable technical assistance.

sterile conditions.[2] Furthermore, Shope (1927) found a bacteriophage active against four species of bacteria, in salt solution extracts of wild, contaminated houseflies. This fact showed that the housefly organism and 'phage are not incompatible.

Fortunately for the work, we possessed cultures of a non-lysogenic although lytically susceptible bacterium. This microörganism is a staphylococcus, *S. muscae*, originally isolated and described (Glaser, 1924 and 1926) from cases of a specific disease of adult houseflies. Since that time the cultures have lost their pathogenicity for flies and instead of invading the body cavity, as was originally the case, now remain exclusively within the alimentary tract. The microörganism grows readily on a variety of artificial media. It is exceedingly susceptible to the bacteriophage isolated from wild houseflies, giving complete lysis up to a dilution of 10^{-12}. Nevertheless, even when originally isolated from the hemolymph of diseased flies, the staphylococcus was 'phage-free and has remained so up to the present time. For these reasons a number of investigators in this laboratory have found *S. muscae* a convenient indicator in their work with bacteriophages.

EXPERIMENTAL

Only houseflies free from bacteria and bacteriophage were used, unless, as in certain experiments, purposeful contaminations were practised.

Table 1 gives the data for an initial experiment with 100 sterilized housefly eggs. A test for bacteriophage against the staphylococcus with the larval medium and the adult food yielded negative results. A similar test against the emerged adults was also negative. In each test with adults, 25 newly emerged flies were ground fine, under aseptic conditions, in 10 cc. of bouillon. This material was incubated for 48 hours, filtered through a Berkefeld "N" candle and the filtrate, in dilutions up to 10^{-12}, tested against the indicator microörganism. In the initial tubes 0.5 cc. of a 48-hour bouillon culture of the staphylococcus was added to 0.5 cc. of the filtrate and the tubes were incubated for 48 hours. Following this procedure six passages from each tube to

[2] The description of a method for rearing houseflies free from microörganisms and bacteriophage has been accepted for publication by the *Journal of Parasitology*.

fresh bouillon were always made, with 48-hour incubation periods, to be certain of the results. Sterile maggots and eggs were also, at times, tested for bacteriophage with negative results. In such cases an extract prepared from 25 maggots or from 1,000 eggs was used. In column four the results of bacterial sterility tests are given and, in column five, the number of adults obtained. This experiment covers a period of three generations and the results were entirely negative for the presence of both bacteria and bacteriophage.

TABLE 1

Aseptically Reared Flies and Their Environment Tested for Bacterial and 'Phage Sterility

Initial experiment	'Phage test against staphylococcus		Bacterial sterility test	No. of emerged flies*
	With medium and food	With emerged flies		
100 sterilized eggs	0	0	0	75
1st generation 3♂ 3♀	0	0	0	60
2nd generation 3♂ 3♀	0	0	0	130
3rd generation 3♂ 3♀	0	0	0	105

* According to Hewitt and others, individual females may deposit 100 to 150 eggs. Since we used three females in our transfers more flies should have emerged than the figures in the table indicate. Crowding and other factors possibly produced this result. However, this does not matter, because the experiments were solely aimed to provide a sufficient number of adults for the tests.

Table 2 shows the data for three separate experiments obtained through eight generations when the sterile breeding medium of sterile flies was experimentally contaminated with a 48-hour bouillon culture of *S. muscae*. Although the staphylococcus was recovered at each generation, no bacteriophage was present. A control experiment (no. 4) was performed in an identical manner with the exception that naturally contaminated flies, reared in horse manure, were employed. In this case, the insects and the breeding medium showed the presence of bacteriophage up to the eighth generation, at which time the experiments were terminated.

In the above outline the sterile, adult flies were tested on emergence. It was thought that perhaps not enough time had intervened to

produce a reaction in the adult forms. As the experiments show, the staphylococcus and its products were in contact with sterile fly tissues through eight generations extending over a period of many weeks. However, it was possible that the adult stage alone, if given more time, might react and produce 'phage. The mature forms do not live very long and under the conditions of the experiments, rarely survived over 13 days. Nevertheless, experiments were performed by contaminating the larval breeding medium with the staphylococcus and testing the adults 4, 8 and 13 days after emergence. The microörganism was recovered in the pure state from all these flies, but tests for 'phage carried through six transfers in bouillon were invariably negative. An

TABLE 2

Attempts to Produce 'Phage in Sterile Flies by Feeding a Non-Lysogenic Staphylococcus

Experiment no.	Age in days of adult flies	Results of 'phage tests on 8 generations of flies	Results of 'phage tests on breeding medium for 6 transfers	Recovery of staphylococcus for 8 generations
1	4	0	0	+
2	8	0	0	+
3	13	0	0	+
Control (with naturally contaminated flies)				
4	4–13	+	+	

extract of wild contaminated adults gave complete lysis of the staphylococcus through the sixth passage in bouillon up to a titer of 10^{-12}.

Flies caught in nature and bred in natural horse manure for eight generations yielded 'phage at each generation of adults. Flies reared under sterile conditions were negative for 'phage at each generation during eight generations. The same was true of sterile flies contaminated with *S. muscae*. This shows that the staphylococcus has not the ability to stimulate the fly organism to form 'phage.

Further experiments showed that 'phage did not survive long in sterile flies unless a living, susceptible bacterium was present. In one set, the maggot medium was artificially contaminated separately with 'phage and with a culture of the staphylococcus killed by subjecting it to a temperature of 65° C for one hour. 'Phage was recovered from

the first crop of adults, but failed to appear again from the second to the conclusion of the experiment at the eighth generation.

In a second set, the medium was purposely contaminated with 'phage alone. 'Phage was again recovered from the first crop of adults, but failed to appear in any of the later generations. In a third set, the medium was experimentally contaminated with 'phage and with a living culture of the microörganism with the result that 'phage was recovered at each generation up to the concluding, eighth crop of adults.

SUMMARY AND CONCLUSIONS

Houseflies caught in nature or bred in the contaminated state invariably harbored bacteriophage. By establishing, in the alimentary tract of sterile houseflies, a non-lysogenic staphylococcus known to be susceptible to lysis, no 'phage was formed. In the absence of this microörganism, 'phage, when given to sterile flies, survived for one generation; whereas, in the presence of the bacterium it persisted for eight generations and probably would have survived indefinitely.

These results do not support the theory that 'phage is the result of the interaction of host and bacteria.

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TAXONOMIC RELATIONSHIPS OF PLANTS SUSCEPTIBLE TO INFECTION BY TOBACCO-MOSAIC VIRUS

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For many years it was thought that tobacco-mosaic virus (tobacco virus 1) could infect only solanaceous plants. Later 2 susceptible species were recognized in other families. These were *Martynia louisiana* Mill. in the family Martyniaceae (1) and *Phaseolus vulgaris* L. in the family Leguminosae (7). In 1934 Grant (2) presented a radically different view of the situation; he infected 29 of 121 tested species in 14 different families among 40 that he investigated. His recognition of wider susceptibility depended on the use of more effective methods of inoculation than had been available for most earlier studies and on observation of symptoms other than systemic mottling. It seemed to the writer that an even larger proportion of tested species might be found susceptible to infection with strains of tobacco-mosaic virus if other methods of determining susceptibility were used to supplement the usual method of observing symptoms after inoculation.

On this account, 2 auxiliary tests were applied to plants of 73 species of herbaceous dicotyledons. Most, but not all, species were subjected to both tests. One of these tests was dependent on quantitative measurement of virus developed at the site of inoculation, the other was dependent on conspicuous local lesions. In the first test, a green-mottling strain of tobacco-mosaic virus was used; in the second, a yellow-mottling strain of the same virus. To date 63 per cent of the 73 species have proved susceptible. These include some that have long been supposed to be immune.

It is the purpose of this paper to present the data derived from the 2 sets of tests. The observations are not primarily of importance as indicating the wide range of disease caused by tobacco-mosaic

virus, since many of the newly demonstrated hosts localize the virus and are little injured by its presence. The results are considered significant rather because they show a close relationship between susceptibility of plants and taxonomic affinities. Perhaps there is a corresponding orderly distribution in nature of some substances or conditions important for increase of tobacco-mosaic virus. The failure of the virus thus far to multiply *in vitro* lends interest to any conceptions with regard to factors allowing or preventing its increase.

MATERIALS AND METHODS

Plants were secured for testing by transplanting a wide variety of dicotyledons found growing as weeds in greenhouses, fields, and woods in the vicinity of Princeton, N. J. Susceptibility tests were performed only on young plants, in a greenhouse held at a temperature of about 22°C. (rarely below 21°C. or above 27°C.). The plants were later grown to maturity for identification of species. Aid in identification was kindly given by members of the staff of The New York Botanical Garden.

The first type of test consisted of quantitative measurement to detect increase of virus at the site of inoculation. For this test all plants were inoculated with typical tobacco-mosaic virus (distorting strain of tobacco-mosaic virus, 4, p. 847; 5, p. 897). Inoculation consisted of rubbing 3 or more leaves of each plant with a cheesecloth pad saturated with expressed juice of mosaic plants of Turkish tobacco (*Nicotiana tabacum* L.), diluted with about 50 parts of water. The rubbed area on the leaves was then further inoculated by making at least 100 pin punctures through the residual inoculum with No. 00 insect pins bound together in a fascicle of 5. Generally, only one plant of each species was tested. The rubbing treatment constituted an inoculation sufficient under the conditions of the tests to produce about 150 primary infections in a comparable leaf area of *N. langsdorffii* Weinm., used as a control on infectivity of inoculum. Pin-puncture inoculation was not thought to be so effective as rubbing inoculation, but it served to mark the inoculated leaves for later identification and furnished a safeguard against the possibility that some species, not susceptible to infection by rubbing, might yet prove susceptible by pin-puncture inoculation.

Ten to 12 days after inoculation, the marked leaves from each plant were separately wrapped in cheesecloth, and crushed. Diluted and undiluted expressed juices from each sample were used to inoculate 5 leaves of a plant of *Nicotiana langsdorffii*. A species was considered susceptible when undiluted juice expressed from inoculated leaves induced the production of more than 10 necrotic primary lesions on the leaves of *N. langsdorffii*. Nearly all the susceptible species produced concentrations of virus sufficient to give far more than this minimum number. A few may have been incorrectly classified by adherence to this arbitrary limit, but it was necessary to use some standard. This seemed a suitable requirement, since experience has shown that residual virus on leaves thus inoculated gives less than this number of lesions. Moreover, of the 3 species giving small but significant numbers of lesions in the subinoculation experiments, 2 showed susceptibility also when the second type of test was applied.

All but 2 of the species not shown to be susceptible by this test for virus increase, together with many of the species known to be susceptible, were subjected to the second type of test. This involved inoculation with a yellow-mottling strain (572 D1) derived recently from the distorting strain of tobacco-mosaic virus. This strain produced yellow-mosaic symptoms in tobacco and in many other susceptible species. The symptoms induced by it have been represented in an earlier paper (5, Fig. 1, a), as typical of those produced by highly invasive yellow-mottling strains of tobacco-mosaic virus. The typical tobacco-mosaic virus used in the first test rarely produced visible local lesions, but the yellow-mottling strain often gave evidence of its increase in the inoculated tissues by production of yellow lesions at the site of inoculation, even in plants in which it did not produce systemic infection. It was necessary to avoid large amounts of virus in the inoculum when typical tobacco-mosaic virus was used, lest residual inoculum interfere with tests for increase of virus. The yellow-mosaic strain, on the other hand, could be applied without dilution, since no subinoculation test was to be made. Through its use, a few species, judged by the tests for virus increase to be doubtfully susceptible, or insusceptible, were shown to be susceptible.

A large part of this work was completed before the description by

Rawlins and Tompkins (8) of their useful method of inoculation with carborundum. A few additional trials were made by this method, but they failed to infect species previously found insusceptible. Future development of better methods for inoculation and for recognizing infection may finally show a larger proportion of species to be capable of supporting increase of tobacco-mosaic virus, but present methods seem to have demonstrated the existence of a naturally insusceptible group.

RESULTS OF EXPERIMENTS

In the accompanying table, the tested species are arranged by families in the order in which they appear in Gray's New Manual of Botany (3). Susceptibility is indicated by the letter S in the 5th column wherever the results of subinoculation tests for virus increase (3rd column) or results of inoculation with the yellow-mottling strain of virus (4th column) justify such a classification. Some of these species were later tested for virus in uninoculated leaves at the tops of the plants. A record of the number of lesions produced by this subinoculation is given with that for the inoculated leaf. It appears after a semicolon and a small letter s, signifying systemic infection. Thus, in the table, *Polygonum hydropiper* 550; s, 0 indicates a plant with a localized infection; in this species much virus was detected in the inoculated leaf after 10 days, but none in uninoculated leaves at the top of the same plant after a month. On the other hand, *Hedeoma pulegioides* 950; s, 930 signifies a plant with systemic infection; in this species much virus was detected in juice expressed from each source.

The distribution of susceptible and insusceptible species in the table suggested the existence of 4 natural groups: 1st, a largely susceptible group, from Polygonaceae to Cruciferae, of 9 families, with 22 susceptible and 2 insusceptible species; 2nd, an almost entirely insusceptible group, from Rosaceae to Umbelliferae, of 11 families, with 2 susceptible and 16 insusceptible species; 3rd, an entirely susceptible group, from Verbenaceae to Rubiaceae, of 5 families, with 13 susceptible and no insusceptible species; and 4th, a group only partly susceptible, comprising the families Lobeliaceae and Compositae, with 9 susceptible and 9 insusceptible species.

Consideration of the tested families from the viewpoint of their probable relationships as represented by Mez and Ziegenspeck (6) discloses associations of susceptible species that are still more striking

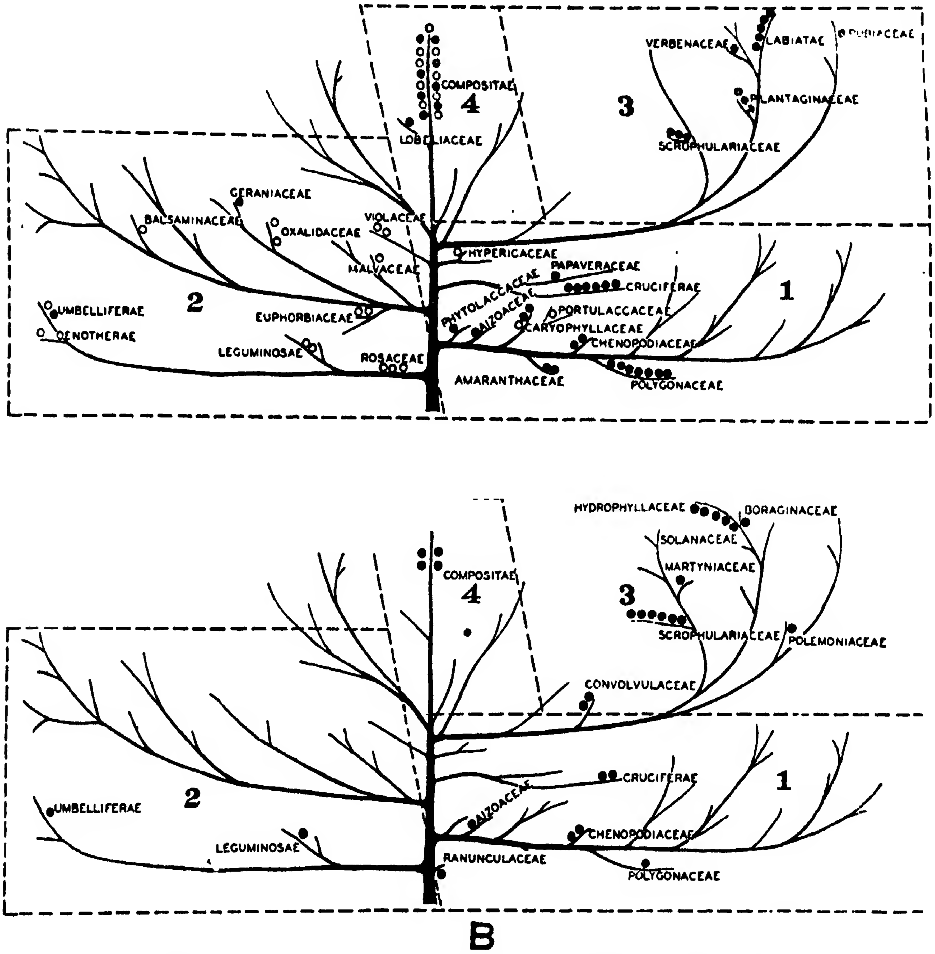

FIG. 1. Taxonomic relationships of plants susceptible to infection by tobacco-mosaic virus. A. Families of tested dicotyledonous plants, diagrammatically arranged according to the plan of the Königsberger Stammbaum of Mez and Ziegenspeck (6). Species tested by inoculation with tobacco-mosaic virus are indicated by circles; black circles indicate susceptible species; white circles, insusceptible species. B. Similar diagram, showing distribution of species found susceptible by Grant in 1934 (2).

than those shown in the linear arrangement of the table. The Königsberger Stammbaum is an evolutionary diagram, constructed by the aid of serological tests that determine affinities of plant species

on the basis of common antigenic constituents. A reduced representation, following the plan of the Königsberg diagram, is shown in figure 1, A. Only families tested in the present study are included; species are indicated by circles (black for susceptible, and white for insusceptible species), and the 4 groups are separated by broken lines. The 1st group of families, mentioned previously as consisting largely of susceptible species, comprises 3 branches on the right of the diagram. The 2nd group of families, consisting mostly of insusceptible species, constitutes 3 branches on the left side. The 3rd group of families, comprising only susceptible species, constitutes a single great branch on the right; this branch carries the Solanaceae, not included in this study, but known to contain many susceptible species. The 4th group, of mixed susceptibility, represents the top of the main stem of the diagram. The 4 groups in the table and the corresponding groups in the diagram differ in but one species, *Hypericum boreale* (Britton) Bicknell of the Hypericaceae, which appears in the 2nd group of the table but in group 1 of the diagram.

The diagrammatic representation shows more clearly than the linear arrangement of the table that susceptible species occur almost entirely in closely associated families (at the right of the diagram), and that insusceptible species are also definitely grouped together (at the left of the diagram). The Compositae, shown at the top of the diagram, are known to have affinities with families at each side; it is not surprising, therefore, that they show susceptibility in some species and not in others.

To summarize the data presented in table 1 and figure 1, A, it may be stated that 46 of the 73 tested species proved susceptible. One of the 46 species (*Daucus carota* L., wild carrot) has been reported previously as being susceptible (2); Grant infected a cultivated form of this species.

For comparison with the distribution of the 46 species here found susceptible, the 29 non-solanaceous hosts originally reported by Grant (2) are shown in figure 1, B; only 2 of these, *Daucus carota* and *Phaseolus vulgaris*, fall into the 2nd group of families shown in the table and diagram. Grant found all 5 tested species of the Hydrophyllaceae to be susceptible; this family was not represented in the writer's tests,

TABLE 1

Arrangement of Tested Species of Plants by Families, with Results of Experiments Performed for Detection of Increase of the Distorting Strain, and for Detection of Symptoms Induced by a Yellow-Mosaic Strain (572 D1) of Tobacco-Mosaic Virus

Family	Species	Lesions on subinoculation; distorting strain	Symptoms observed; yellow-mosaic strain 572 D1	Susceptibility
Polygonaceae	*Rumex crispus* L.	550	Yellow primary lesions; red peripherally	S
	" *acetosella* L.	350	None	S
	Polygonum aviculare L.	600	Large elongated yellow primary lesions	S
	" *erectum* L.	180	Elongated yellow primary lesions	S
	" *persicaria* L.	290; s, 2	None	S
	" *hydropiper* L.	550; s, 0	None	S
	" *convolvulus* L.	570	Yellow primary lesions, elongated along veins	S
Chenopodiaceae	*Chenopodium ambrosioides* L.	900	Necrotic primary lesions	S
	" *album* L.	250	Small yellow primary lesions, becoming necrotic	S
Amaranthaceae	*Amaranthus hybridus* L.	6	Yellow primary lesions	S
	" *graecizans* L.	31	Yellow primary lesions, becoming semi-necrotic	S
Phytolaccaceae	*Phytolacca decandra* L.	2	Yellow primary lesions on mature leaves only	S
Aizoaceae	*Mollugo verticillata* L.	500	Yellow primary lesions, becoming necrotic	S
Caryophyllaceae	*Stellaria media* (L.) Cyrill.	0	Few yellow primary lesions	S
	Cerastium vulgatum L.	700	Zonate yellow primary lesions, becoming necrotic	S
	Lychnis alba Mill.	5; s, 0	None	..
Portulacaceae	*Portulaca oleracea* L.	0	None	..
Papaveraceae	*Chelidonium majus* L.	570; s, 0	Diffuse yellow primary lesions	S

TABLE 1—*Continued.*

Family	Species	Lesions on subinoculation; distorting strain	Symptoms observed; yellow-mosaic strain 572 D1	Susceptibility
Cruciferae	*Lepidium ruderale* L.	330; s, 90	Not tested	S
	" *campestre* (L.) R. Br.	740; s, 0	Yellow primary lesions extending down veins	S
	Capsella bursa-pastoris (L.) Medic.	600	Yellow primary lesions	S
	Sisymbrium officinale (L.) Scop.	65	Multizonate yellow primary lesions	S
	" *thalianum* (L.) J. Gay	525	Not tested	S
	Radicula palustris (L.) Moench.	180; s, 0	None	S
Rosaceae	*Potentilla arguta* Pursh	0; s, 0	None	..
	" *monspeliensis* L.	0; s, 0	None	..
	Geum canadense Jacq.	0; s, 0	None	..
Leguminosae	*Trifolium repens* L.	0	None	..
	Medicago sativa L.	4; s, 0	None	..
Oxalidaceae	*Oxalis stricta* L.	0	None	..
	" *corniculatus* L.	0	None	..
Geraniaceae	*Geranium carolinianum* L.	39	Yellow primary lesions and systemic yellow mottling	S
Euphorbiaceae	*Acalypha virginica* L.	3	None	..
	Euphorbia maculata L.	0; s, 0	None	..
Balsaminaceae	*Impatiens biflora* Walt.	9; s, 0	None	..
Malvaceae	*Malva rotundifolia* L.	0; s, 0	None	..
Hypericaceae	*Hypericum boreale* (Britton) Bicknell	0; s, 0	None	..
Violaceae	*Viola papilionacea* Pursh	0	None	..
	" *sagittata* Ait.	0; s, 0	None	..
Onagraceae	*Circaea lutetiana* L.	0; s, 0	None	..
Umbelliferae	*Sanicula canadensis* L.	0	None	..
	Daucus carota L.	450	None	S

Verbenaceae	*Verbena urticaefolia* L.	44; s, 0	None	S
Labiatae	*Prunella vulgaris* L.	275; s, 0	Yellow primary lesions	S
	Lamium amplexicaule L.	450	Yellow primary lesions	S
	Hedeoma pulegioides (L.) Pers.	950; s, 930	Systemic yellow mottling	S
	Lycopus virginicus L.	135	None	S
	" *rubellus* Moench.	86; s, 0	None	S

TABLE 1—*Concluded.*

Family	Species	Lesions on subinoculation; distorting strain	Symptoms observed; yellow-mosaic strain 572 D1	Susceptibility
Scrophulariaceae	*Linaria vulgaris* Hill	400	Zonate yellow primary lesions	S
	Veronica officinalis L.	0	Few zonate yellowish green primary lesions	S
	" *peregrina* L.	56	None	S
Plantaginaceae	*Plantago major* L.	400	Necrotic primary lesions	S
	" *rugelii* Dcne.	81; s, 0	Yellow primary lesions, becoming necrotic	S
	" *lanceolata* L.	100	Yellow primary lesions, becoming semi-necrotic	S
Rubiaceae	*Galium triflorum* Michx.	19; s, 0	None	S
Lobeliaceae	*Lobelia inflata* L.	65	None	S
Compositae	*Solidago rugosa* Mill.	0; s, 0	None	..
	" *graminifolia* (L.) Salisb.	0; s, 0	None	..
	Aster ericoides L.	0; s, 0	Not tested	..
	" *multiflorus* Ait.	0; s, 0	Not tested	..
	" *lateriflorus* (L.) Britton	0; s, 0	None	..
	" *dumosus* L.	390; s, 0	Not tested	S
	Erigeron annuus (L.) Pers.	255; s, 0	Yellow primary lesions	S
	" *canadensis* L.	1	Yellow lesions, becoming necrotic	S
	Ambrosia artemisiifolia L.	17	Few yellow primary lesions	S
	" *trifida* L.	13; s, 0	Few yellow primary lesions	S
	Rudbeckia hirta L.	0; s, 0	None	..
	Helianthus tuberosus L.	0; s, 0	None	..
	Bidens frondosa L.	430; s, 0	Yellow primary lesions	S
	Galinsoga parviflora Cav.	270	Yellow primary lesions, elongated along veins	S
	Cirsium arvense (L.) Scop.	5; s, 0	None	..
	Taraxacum officinale Weber	1	None	..
	Hieracium scabrum Michx.	0; s, 0	Few yellow primary lesions	S

but it, like the largely susceptible family Solanaceae, belongs to the 3rd group, in which all tested species proved susceptible.

DISCUSSION

Substances and conditions requisite for increase of tobacco-mosaic virus are evidently more widespread in herbaceous dicotyledons than has been previously recognized, 63 per cent of the tested species in all families proving susceptible. If the species of plants in the 11 families referred to as constituting the largely insusceptible group 2 are excluded from consideration, 80 per cent of the remaining species are susceptible. If the moderately susceptible group 4 also is eliminated, 95 per cent of the remaining species (35 of 37) are susceptible; these species are distributed in 14 related families. Recognition of so large a proportion of susceptible species as here reported is dependent on the use of 2 tests for virus increase *at the site of inoculation*. Exclusive use of tests for *systemic spread* of virus, including measurement of virus increase and observation of symptoms, permits the recognition of far fewer susceptible species.

Localization of virus within the inoculated leaf was found to be a very common phenomenon among the tested species. It is probable that systemic invasion by tobacco-mosaic virus occurs in only a minority of susceptible species among flowering plants in general.

It is often desirable to obtain virus from pairs of species not closely related to each other, since unrelated hosts are not likely to possess identical antigenic compounds or proteins. If hosts are selected from families in group 3 of the table on the one hand, and from families in groups 1 or 4 on the other hand, numerous pairs of susceptible, but distantly related, plants may be obtained. Some of the chosen species may localize the virus, but objection to their use need not be raised, even though traces of inoculum from the original host plant may be considered undesirable in extracted juice. The difficulty can be avoided by a series of transfers from leaf to leaf in the new species, to eliminate all previous constituents of the original host plant, except the tobacco-mosaic virus, itself.

SUMMARY

Among 73 tested species of herbaceous dicotyledons, about two thirds (46) proved susceptible to infection with tobacco-mosaic virus.

The criteria of susceptibility were detection of increase of virus in inoculated leaves by quantitative subinoculation tests and observation of symptoms at site of inoculation. A correlation seemed to exist between accepted taxonomic classification and susceptibility, since almost all of the tested species in one group of 11 families apparently lacked ability to support increase of tobacco-mosaic virus, whereas 95 per cent of the species in another group of 14 families were shown to have this ability.

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SEASONAL VARIATIONS IN SUSCEPTIBILITY OF TOBACCO TO INFECTION WITH TOBACCO-MOSAIC VIRUS

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In connection with experimental studies on the effect of mineral nutrition on host susceptibility to infection with tobacco-mosaic virus, the question arose as to whether this susceptibility is influenced also by fluctuations in environmental conditions due to seasonal changes. The object of this paper is to report briefly the results of 129 consecutive weekly tests that were carried out in an endeavor to answer this question.

MATERIALS AND METHODS

Seedlings of Turkish tobacco, *Nicotiana tabacum* L., were cultured in white quartz sand until they had developed 3 or 4 leaves and were about 2 cm. in height. Once each week 126 uniform plants at this stage of growth were transplanted into 4-inch porous clay pots, filled with composted soil, to which peat moss had been added. These pots were then placed in loose peat moss in a side bench in a greenhouse. One week the pots were placed to the left of the middle of the bench, the next week to the right, the position of the plants on the bench thus being alternated throughout the experiment.

On the 3rd day after potting, 100 plants were inoculated by means of a single puncture with a needle, size 00, in the youngest leaf over 0.5 cm. long on each plant. This method was used so that relatively uniform doses of inoculum might be introduced into each plant. The inoculations were made late in the afternoon to minimize any possible harmful effect of unfavorable light or temperature on the freshly inoculated leaf. The extract of green tobacco-mosaic virus (Johnson's tobacco virus 1) was prepared as follows: Undiluted juice from

diseased tobacco plants was frozen for 48 hours in test tubes and then thawed. It was carefully remixed and 2-cc. portions placed in small test tubes. These tubes were tightly corked, sealed with paraffin, and stored in a cold room at $-14°$ C. until used. Each week the juice in one tube was thawed, used for the inoculation, and then discarded. In this way the virulence of virus extract was held as uniform as has been found possible. It is probable, therefore, that variations in the number of plants becoming diseased at different seasons were due to variations in host suceptibility rather than to changes in the inoculum.

The following observations and calculations were made each week: (1) incubation period of the disease, (2) number of plants infected, (3) greenhouse temperature, and (4) hours of sunshine. The temperature record was made by means of a "Tycos" recording thermometer placed on the greenhouse bench beside the test plants. The record of the hours of sunshine was obtained from a substation of the Weather Bureau of the United States Department of Agriculture, located at Trenton, N. J., 12 miles from Princeton. Some variations occurred between the number of hours of sunshine in Trenton and in Princeton, but periodic checks indicate these to be quite small.

EXPERIMENTAL

The susceptibility of the young plants, measured by the percentage of plants that showed symptoms of tobacco mosaic, is expressed graphically in figure 1. Each circle on the curve represents the percentage of infection in a single test. From this graph it is apparent that variations in the number of plants becoming diseased were obtained from week to week. The cause of this variation is difficult to define because of the complex interrelationship of the environmental factors involved. Of these factors, the following are probably the most important: temperature, light intensity and duration, and humidity. A detailed study of these factors was not attempted because of limited equipment, but such observations as were possible with the facilities available were made on each test.

In an effort to illustrate the influence that some of these factors might exert on susceptibility, the graphs in figure 2 have been prepared. Each circle on these 4 curves represents the average of the

weekly observations for each 2-month period. The upper graph shows the average hourly temperature in the greenhouse during the day of inoculation and the day following in each test. The second graph shows the average daily hours of sunshine during the same period. The third graph shows the average number of days that elapsed from time of inoculation to the appearance of symptoms of the disease. The lowermost graph shows the average susceptibility of the plants as determined by the percentage of plants that became diseased.

From the graph expressing susceptibility, it is apparent that the plants showed a somewhat definite annual cyclic variation in their

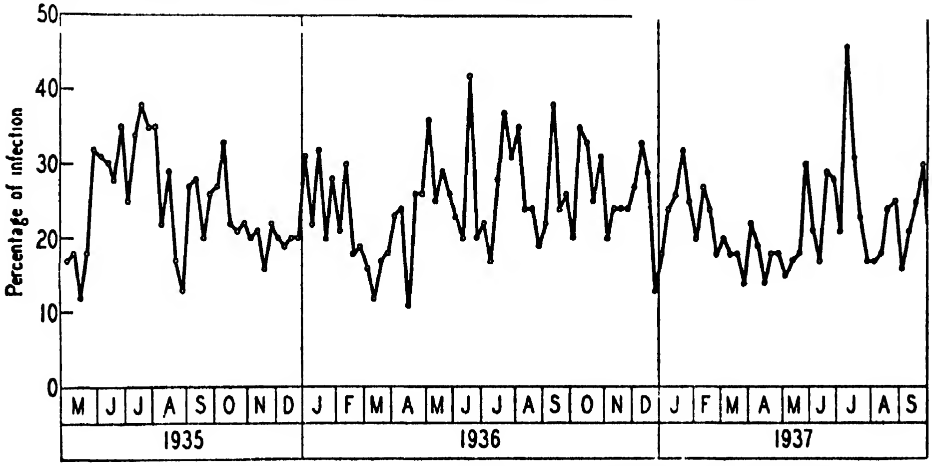

FIG. 1. Susceptibility of young tobacco plants tested at weekly intervals to infection with tobacco-mosaic virus. Each circle represents the percentage of 100 plants which became infected in each test.

susceptibility to infection with tobacco-mosaic virus. In both years the susceptibility was low during late winter and early spring, and high during early summer. In 1935, susceptibility was high during June and July and then decreased about 25 per cent during August and September. The average susceptibility remained fairly constant at this level until February, 1936, when a second decrease was observed. Following an increase in susceptibility in April–May, 1936, no appreciable differences were observed until in February, 1937, when a decrease of about 20 per cent occurred. In 1937 susceptibility decreased gradually from its maximum in June and July.

In an effort to correlate to some extent these variations in suscepti-

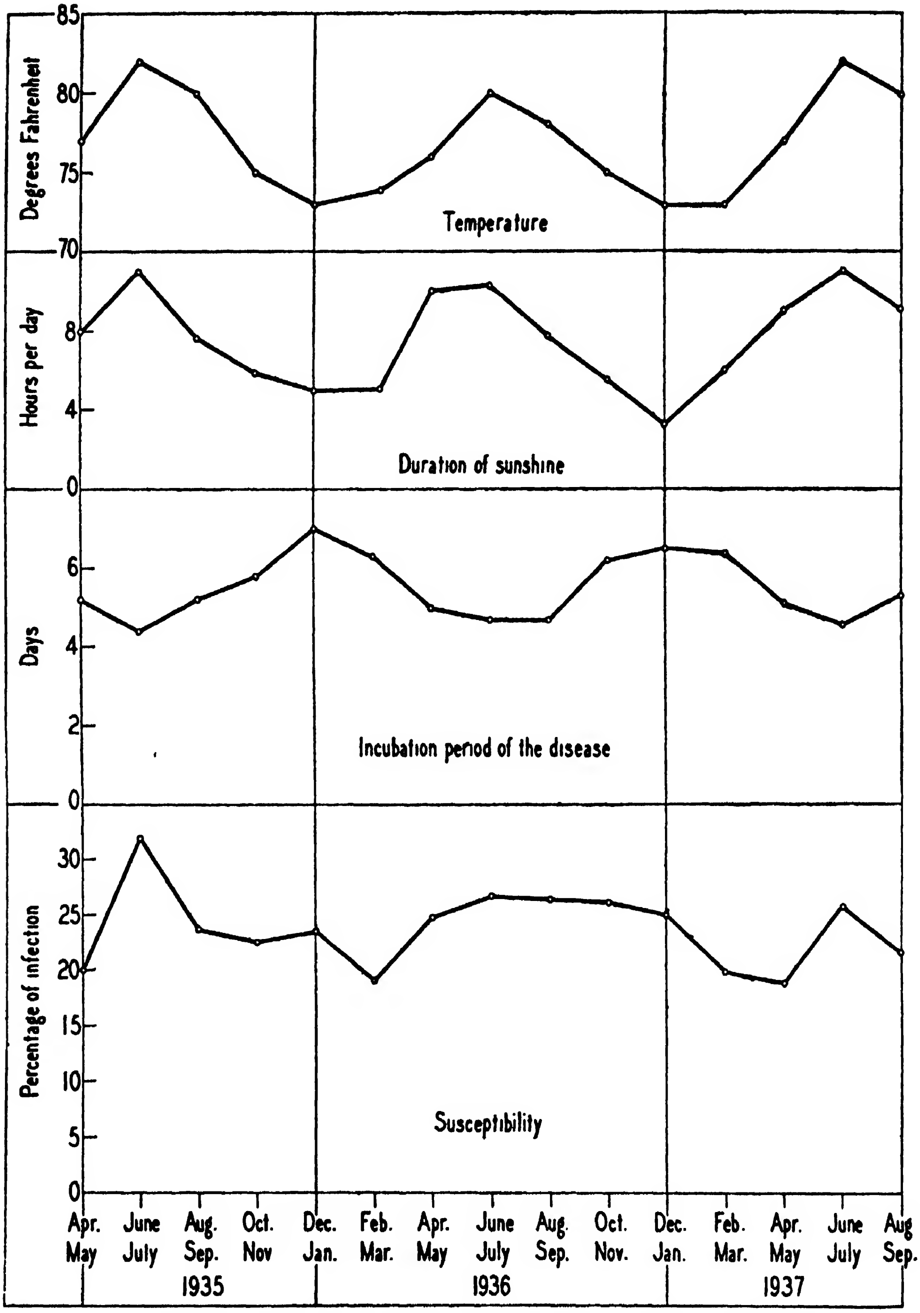

FIG. 2. Graphs showing the relation of temperature and hours of sunshine to incubation period of the disease and the susceptibility of the host. Each circle represents the average of the weekly observations made during each 2-month period.

bility with seasonal fluctuations in temperature and light, certain salient features in the graphs in figure 2 may be mentioned. The period during which plants were low in susceptibility did not coincide with that in which the average temperature was lowest or the duration of sunshine was shortest, but followed this period by at least 2 months. The lowest average temperature and the shortest duration of sunshine occurred in the December–January period each year, but the plants were lowest in susceptibility during February–March in 1936 and during April-May in 1937. However, in both years the period of high susceptibility coincided with that of high temperature and long duration of sunshine.

The incubation period of the disease showed very marked variations from season to season, and these variations seemed constant from year to year. During June and July the symptoms of the disease appeared about 4 or 5 days after inoculation. During December and January, however, at least 6 or 7 days elapsed before the appearance of the disease. These variations seemed to be definitely correlated with seasonal fluctuations in light and temperature. The incubation period was shortest during June and July, a period characterized by high temperature and long duration of sunshine. Moreover, during December and January when the incubation period was long, the average temperature was lower and the duration of sunshine shorter than at any other time during the year.

The general results of the experiments reported here indicate that the normal variations in host reaction to tobacco-mosaic virus from season to season should be considered in planning certain types of investigations. For example, experiments in which high susceptibility to infection is desirable should be carried out during the summer months. Furthermore, the length of the incubation period of the disease is an important factor in studies pertaining to virus movement. In reporting experiments dealing with the incubation period of the disease, mention should always be made of the seasons in which such experiments were carried out.

SUMMARY

A study involving weekly tests over a period of 2½ years showed definite variations in the susceptibility of small Turkish tobacco

plants to infection with tobacco-mosaic virus. Susceptibility was high during early summer, a period characterized by high temperature and long duration of sunshine, and low during late winter and early spring. The incubation period of the disease within the plant showed a direct correlation with seasonal fluctuations in light and temperature, being short during early summer and long during the winter months.

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THE INFLUENCE OF MINERAL NUTRITION ON THE REACTION OF SWEET-CORN SEEDLINGS TO PHYTOMONAS STEWARTI

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(Accepted for publication, November 3, 1937)

Several workers (1, 2, 6, 7, 8, 10) have observed that the reaction of certain plants to infectious agents may be altered by varying the mineral composition of the nutrient solution. In connection with experiments being conducted in this laboratory with bacterial wilt of maize, the causal agent of which is *Phytomonas stewarti* (E. F. S.) Bergey *et al.* (*Bacterium stewarti* E. F. S.), it seemed desirable to ascertain whether the host-parasite relationship of this disease could be modified by mineral nutrition. A study was undertaken, therefore, to determine the influence of nitrogen, phosphorus, and potassium nutrition on the reaction of sweet-corn seedlings to *P. stewarti*. The responses shown by the seedlings are reported in this paper.

Materials and Methods

Sweet-corn seedlings of the variety Golden Bantam were grown in white quartz sand previously washed with tap water. Five seeds were placed in each 4-inch porous clay pot, covered with a layer of sand about one-half inch in depth, and kept moist with tap water. When the seedlings had reached a height of 3 or 4 cm., usually 8 days after planting, they were thinned to 3 seedlings in each pot. Twenty-five pots were started for each nutrient treatment, but at time of inoculation 5 pots in each treatment were eliminated. The pots, set in saucers, were so placed on a greenhouse bench that all were exposed to approximately the same environmental conditions.

Nutrient treatments were started 7 or 8 days after planting. Each pot received 100 cc. of nutrient solution 3 times a week. The composition of the solutions is given in table 1. The salt proportions in

these solutions were based on solution $T_3R_2C_2$ of the Jones-Shive series (3). This solution was chosen because it had produced good growth of corn in preliminary tests. In addition to the salts listed in table 1, boron as H_3BO_3 and manganese as $MnSO_4 \cdot 2H_2O$ were added to all solutions in concentrations equivalent to 0.5 p.p.m. of each element. Sufficient $FeSO_4$ was added to prevent chlorosis from iron deficiency. No attempt was made to hold the osmotic concentration of the solutions constant. The concentrations of nitrogen, phosphorus, and potassium were varied in separate experiments. When the concentration of one of these elements was varied, the concentrations of the other two were held constant.

TABLE 1

Composition of Nutrient Solutions Used in the Different Nitrogen, Phosphorus, and Potassium Experiments

Stock solutions (0.5 molar)	Volume of stock solutions per liter of nutrient solution in experiments on		
	Nitrogen	Phosphorus	Potassium
	cc.	*cc.*	*cc.*
KH_2PO_4	6.3	...	...
$MgSO_4$	7.1	7.1	7.1
$Ca(NO_3)_2$	...	2.9	2.9
$CaCl_2$	2.9	...	...
$(NH_4)_2SO_4$	...	2.8	...
$NH_4H_2PO_4$	...	...	5.6
NH_4NO_3	0–142.9	...	...
NaH_2PO_4	...	0–161.3	0.7
K_2SO_4	...	3.2	0–76.7

The seedlings were inoculated 2 or 3 days after the first application of nutrients. A virulent, single-colony isolate (B–1011), described by McNew (5), was used as the stock culture. The culture was maintained on nutrient-dextrose agar slants at 20°C. and subinoculated into 200 cc. of nutrient broth (Difco), which had previously been supplemented with 0.5 per cent dextrose and adjusted to a pH of 6.8 to 7.0. After incubating for at least 72 hours, the culture was injected into the crown and leaf whorl of 30 seedlings in each group. The 30 seedlings remaining in each group were similarly injected with the

sterile filtrate obtained by twice filtering the culture through Berkefeld "W" cylinders. These injections subjected all seedlings to the same inoculation wounding and to such metabolic by-products of the bacterium as might be present in the culture.

About 10 days after inoculation the total number of leaves, the number of killed leaves, and the number of necrotic lesions on each plant were recorded. The severity of infection was expressed as an infection index, based upon the average number of necrotic lesions per leaf. Since some leaves collapsed from a diffuse wilt or a coalescence of lesions, an accurate count of the number of necrotic lesions on these collapsed leaves was impossible. Each collapsed leaf, therefore, was arbitrarily classified as having 3 lesions. This calculated number of lesions on the collapsed leaves was added to the number of lesions actually recorded and the sum divided by the total number of leaves. The quotient so obtained was designated as the infection index.

After 18 or 20 days of nutrient treatment, the green weights of leaves and stems were determined. Dry weights were determined after the samples had been dried to constant weight at 100°C. in an electric oven.

Each nitrogen, phosphorus, and potassium experiment was carried out independently, since sufficient greenhouse space was not available to conduct more than one at a time. Therefore, the results of one experiment can not be directly compared with those of another, because of possible changes due to environmental factors. However, the experiments were all carried out in the same manner and for approximately the same duration of time, the nitrogen and phosphorus experiments in November and the potassium experiment in the preceding September. Each experiment was repeated at least once, with compatible results.

EXPERIMENTAL RESULTS

Influence of Nitrogen Supply on Host Reaction

The influence of variations in the nitrogen supply on the reaction of corn seedlings to *Phytomonas stewarti* was studied in the first experiment. The basic nutrient solution is described in table 1. Nitrogen was added as NH_4NO_3 in amounts ranging from 0 to 200 mg. of nitrogen per 100 cc. of solution. In all treatments the phosphorus concen-

tration was held constant at 12.5 mg. and the potassium concentration at 10.0 mg. per 100 cc. of solution.

From the experimental data recorded in table 2, it is obvious that nitrogen supply influenced very markedly the growth of corn seedlings in sand. Relatively low nitrogen levels (20–30 mg.) favored the maximum growth of seedlings. As the nitrogen level was raised above 30 mg., the seedlings made only limited growth. Representative seedlings of 3 different treatments were photographed 10 days after inoculation with the sterile filtrate of the culture. These seedlings,

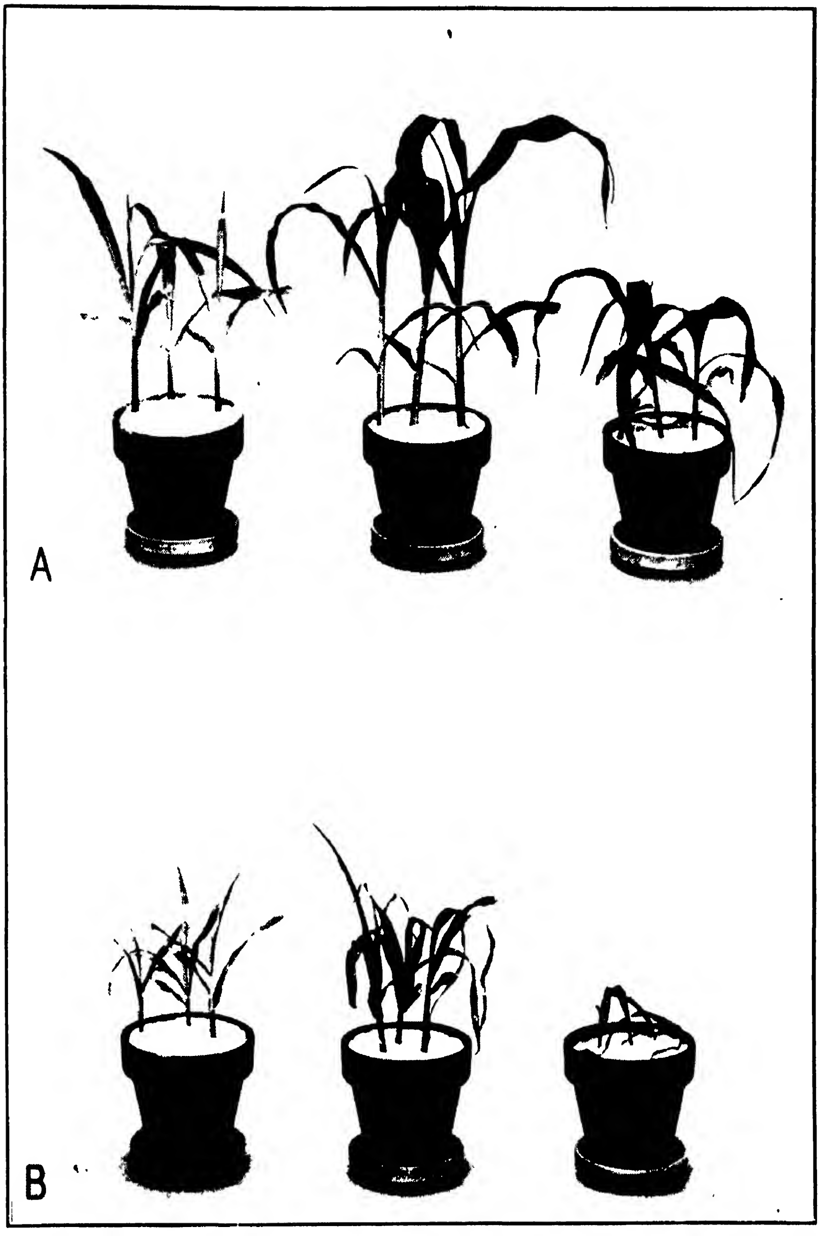
TABLE 2

Influence of Nitrogen Supply on Growth and Reaction of Corn Seedlings to Infection with Phytomonas stewarti

Nitrogen added	Average dry weight per plant		Reaction of diseased seedlings				
			Number of leaves				
	Healthy	Infected	Total	Invaded		Number of lesions	Index of infection
				Living	Dead		
mg./100 cc.	*gm*	*gm.*					
0	.19	.16	124	29	1	47	.40
10	.40	.28	141	57	1	79	.58
20	.43	.23	119	46	5	63	.66
30	.45	.19	140	61	2	93	.71
40	.42	.25	123	65	5	108	1.00
70	.36	.23	131	58	5	109	.95
100	.32	.17	127	70	6	113	1.03
150	.28	.15	117	62	15	106	1.29
200	.19	.10	109	57	15	92	1.26

shown in figure 1, A, had received 0, 40, and 200 mg. of nitrogen, respectively, 3 times each week. The seedlings that had received no nitrogen were light yellowish-green and made very little growth. The lower leaves became shriveled and dry. Slight injury, due unquestionably to nitrogen toxicity, was noticeable in seedlings that had received 200 mg. of nitrogen 3 times each week. This injury was apparent as a wilting at the tips of older leaves. Some of these leaves later became entirely flaccid.

The data in table 2 on the reaction of the seedlings to the disease show that infection became more severe as the concentration of nitro-



Photograph by J. A. Carlile

FIG. 1. Typical corn seedlings that had received 0, 40, and 200 mg. of nitrogen, respectively, 3 times each week, photographed after 2 weeks of nutrient treatment. A. Healthy seedlings. B. Infected seedlings 10 days after inoculation.

gen increased. When more than 150 mg. of nitrogen were added per 100 cc. of solution, severity of infection remained fairly constant. Figure 1, B, shows infected seedlings of the same age and of the same nutrient treatment as those in figure 1, A. In the absence of nitrogen, seedlings showed primary invasion, but little or no secondary invasion. The necrotic lesions produced were small and few in number. None of the nitrogen-deficient seedlings wilted. As the nitrogen level of the nutrient solution was raised, the pathogen produced larger and more numerous necrotic lesions, and caused a wilted condition of many of the invaded leaves. At the higher nitrogen levels, this type of wilting was so intense that about one-half of the inoculated seedlings died within 2 weeks after inoculation. Thus, it is evident that infection was more severe at high nitrogen levels than at levels more favorable for plant growth. The possible significance of these results will be discussed later.

Influence of Phosphorus Supply on Host Reaction

In the second experiment, the influence of variations in the phosphorus supply was studied. The phosphorus levels of the solutions ranged from 0 to 250 mg. per 100 cc. of solution. The composition of the basic solution is given in table 1. The required amounts of phosphorus were added as NaH_2PO_4. In all solutions the nitrogen concentration was held constant at 8 mg. and the potassium concentration at 12.5 mg. per 100 cc. of solution.

The experimental data on the relation between phosphorus supply and growth of corn seedlings are given in table 3. These data show that the plants grew poorly when more than 60 mg. of phosphorus were added 3 times each week. Maximum growth of seedlings was obtained when 20 to 60 mg. were added 3 times each week. Figure 2, A, shows pots of representative seedlings that had received 0, 100, and 200 mg. of phosphorus, respectively, 3 times each week for 3 weeks. Seedlings were injured somewhat by solutions containing either a deficiency or an excess of phosphorus. The seedlings that had received a solution deficient in phosphorus were purplish, with some yellowing apparent on a few of the lower leaves. These seedlings were slightly smaller than those that had received small amounts of phosphorus. The seedlings that had received the largest amounts of

phosphorus grew poorly, but the only noticeable injury was a wilting at the tips of the lower leaves.

Figure 2, B, shows infected seedlings, on the same nutrient treatments as those in figure 2, A. At low phosphorus levels infection was characterized by the formation of definite necrotic lesions. At the higher phosphorus levels infection was manifested not only by necrotic lesions but also by dwarfing of the seedlings and a general wilting of the invaded leaves. This wilting was not intensified to the extent that death resulted within 2 weeks following inoculation, as was the

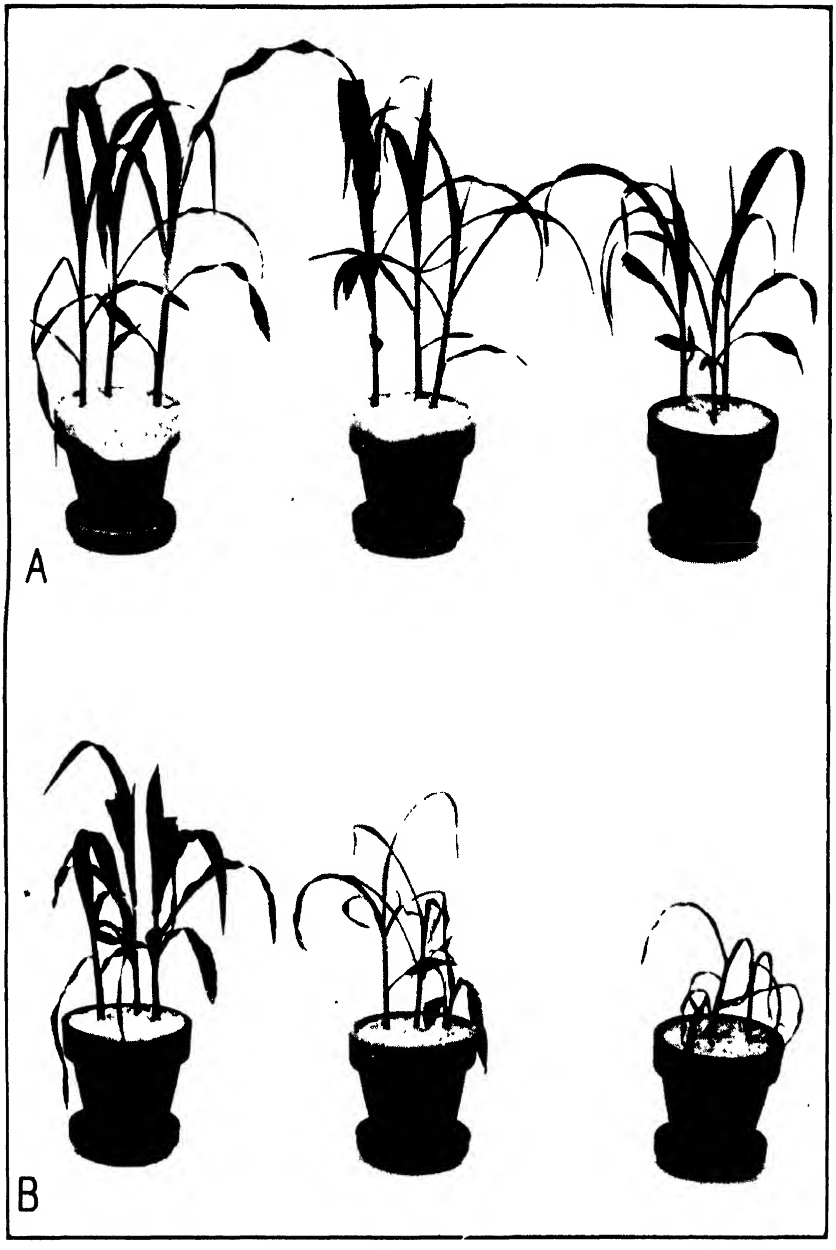
TABLE 3

Influence of Phosphorus Supply on Growth and Reaction of Corn Seedlings to Infection with Phytomonas stewarti

Phosphorus added	Average dry weight per plant		Reaction of diseased seedlings				
			Number of leaves			Number of lesions	Index of infection
				Invaded			
	Healthy	Infected	Total	Living	Dead		
mg /100 cc.	*gm.*	*gm.*					
0	.34	.25	118	60	0	96	.81
20	.41	.25	115	68	2	109	1.00
40	.39	.32	122	73	1	121	1.02
60	.42	.29	121	69	2	125	1.08
80	.35	.28	117	60	3	123	1.13
100	.33	.25	113	66	4	126	1.22
150	.32	.24	113	67	5	116	1.16
200	.28	.19	110	68	4	128	1.27
250	.26	.17	103	63	8	121	1.41

case with the high-nitrogen seedlings. The infection gradually became more severe with increasing amounts of phosphorus, even though these high levels of phosphorus retarded seedling growth.

Since phosphorus was added as NaH_2PO_4, the sodium concentration increased simultaneously with that of phosphorus. In order to determine whether the increased severity of wilt at high levels of NaH_2PO_4 was due to phosphorus or to sodium, an experiment was carried out in which the sodium concentration was held constant at all phosphorus levels by adding the required amounts of sodium as Na_2SO_4. The results from this experiment were comparable with those recorded in



Photograph by J. A. Carlile

Fig. 2. Typical corn seedlings that had received 0, 100, and 200 mg. of phosphorus, respectively, 3 times each week, photographed after 3 weeks of nutrient treatment. A. Healthy seedlings. B. Infected seedlings 16 days after inoculation.

table 3, in that infection was more severe at the higher levels of phosphorus. However, seedlings that received sodium but no phosphorus were somewhat more severely infected than those that received a deficiency of both sodium and phosphorus. These findings indicate that the differences in the host-parasite association brought about by the addition of increasing amounts of NaH_2PO_4 were due primarily to the variations in the phosphorus levels and not to the variations in sodium levels.

TABLE 4

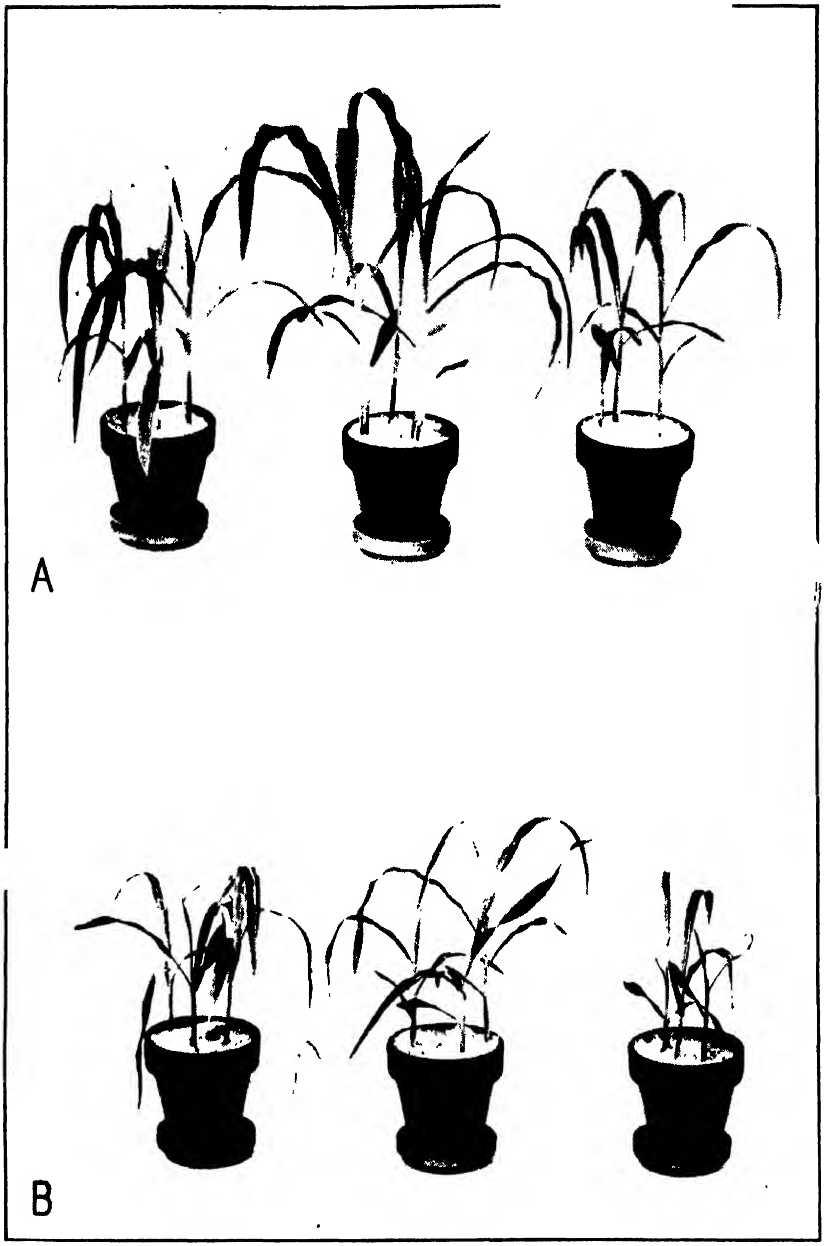
Influence of Potassium Supply on Growth and Reaction of Corn Seedlings to Infection with Phytomonas stewarti

Potassium added	Average dry weight per plant		Reaction of diseased seedlings				
			Number of leaves			Number of lesions	Index of infection
	Healthy	Infected	Total	Invaded			
				Living	Dead		
mg./100 cc.	*gm.*	*gm.*					
0	.44	.36	172	98	13	194	1.35
20	.56	.45	172	82	10	134	.95
40	.61	.39	170	66	14	105	.86
60	.67	.37	165	70	16	122	1.03
80	.59	.43	174	80	12	143	1.03
100	.61	.39	162	69	18	119	1.07
150	.58	.43	167	74	16	135	1.10
200	.49	.30	143	67	27	121	1.41
250	.47	.34	144	63	27	108	1.31
300	.41	.26	131	56	17	94	1.11

Influence of Potassium Supply on Host Reaction

In the third experiment, the influence of potassium supply was studied. The composition of the basic solution is given in table 1. Potassium was added as K_2SO_4 in amounts ranging from 0 to 300 mg. per 100 cc. of solution. In all solutions the nitrogen concentration was held constant at 8 mg. and the phosphorus at 10 mg. per 100 cc. of solution.

The experimental data given in table 4 show that growth of the healthy seedlings increased as the concentration of potassium increased to a level of 60 mg. per 100 cc. of solution. Growth was



Photograph by J. A. Carlile

FIG. 3. Typical corn seedlings that had received 0, 80, and 200 mg. of potassium, respectively, 3 times each week, photographed after 3 weeks of nutrient treatment. A. Healthy seedlings. B. Infected seedlings 16 days after inoculation.

retarded as the concentration was increased beyond this point. Infected seedlings that had received between 20 and 150 mg. of potassium per 100 cc. of solution showed little variation in growth. Figure 3 shows representative healthy and infected seedlings that had received 0, 80, and 200 mg. of potassium 3 times each week for 3 weeks.

Plants that received no potassium showed distinct symptoms of potassium deficiency. The older leaves of healthy plants showed a necrosis, which spread from the tip along the margins to the leaf sheath. The young leaves of healthy seedlings were apparently normal, whereas the young leaves of infected seedlings showed a very marked chlorosis. Definite symptoms of toxicity appeared on all seedlings that had received high levels of potassium. The young whorls failed to develop normally, and they gradually became necrotic. The older leaves became light brown and necrotic. In all treatments where toxicity occurred, the toxicity symptoms were more severe on the infected seedlings than on the healthy seedlings. When 200 mg. of potassium were added 3 times each week, irregular blotches developed on leaves of the infected seedlings but not on those of the healthy seedlings. The same condition with regard to necrosis of the leaf whorls was noticeable at the next higher potassium level (250 mg.). At the highest potassium level (300 mg.), more leaf whorls were injured in infected seedlings than in healthy seedlings.

The data on disease response show that potassium-deficient seedlings were very severely infected, whereas seedlings that had received 20 or 40 mg. of potassium per 100 cc. of solution were only slightly infected. However, the infection became more severe as the potassium level was raised from 40 mg. to 200 mg. These findings with regard to potassium deficiency are very striking and are apparently significant, since 2 subsequent repetitions of this experiment revealed the same relationship. The type of necrotic lesions formed as the result of inoculation appears to be similar in all treatments. Wilting of invaded leaves was more prevalent at the higher potassium levels, but none of the seedlings died as a result of infection in the 14-day period following inoculation.

DISCUSSION

The average number of necrotic lesions per leaf should serve as a satisfactory measure of the host reaction of the pathogen, since corre-

sponding host tissues in all treatments were exposed to the bacterial culture. The infection index, as used in the experiments reported above, is an approximate measure of the number of leaf veins that necrose because of invasion by *Phytomonas stewarti*. Since the severity of infection usually was noted 10 days after inoculation, the infection index, as used here, is essentially a measure of primary infection. Seedlings with low infection indexes suffered very little secondary invasion and generally recovered.

It has been reported frequently that an actively growing host is more susceptible to disease than a slow-growing host. Trelease and Trelease (9) have shown, however, that some condition, or conditions, apparently independent of host vigor appears to determine the susceptibility of wheat to mildew (*Erysiphe graminis* DC.). A similar situation seems to exist in the host-parasite complex of bacterial wilt of maize, as shown by the experimental evidence here reported. For example, seedlings that received nitrogen or phosphorus at concentrations optimum for plant growth were more severely infected than those deprived of the element in question. On the other hand, seedlings that received small amounts of potassium grew better but were less severely infected than those deficient in potassium.

An increase in the concentration of nitrogen, phosphorus, or potassium beyond that optimum for plant growth, as judged by dry weight, increased the severity of the disease. It is probable that the excessive wilting produced by these treatments is due partly to high osmotic pressure of the solution and partly to invasion by the bacteria. The quantity of water available for the leaves apparently is reduced not only by a plugging of the tracheal tubes by the organism, but also by the increased osmotic concentration of the solution.

The effect produced by different amounts of nitrogen, phosphorus, or potassium on the reaction of corn seedlings to *Phytomonas stewarti* may be due to a change in (1) the composition of the host cell so as to alter its susceptibility to parasitic attack, (2) the rate of multiplication of the pathogen, or (3) the virulence of the pathogen within the host. From the experimental evidence presented in this paper, it is not known which of these changes has been instrumental in producing the observed reaction.

If the nutrient solution affected the pathogen directly, the action would undoubtedly be brought about through a change in the com-

position of the transpiration stream. Lowry, Huggins, and Forrest (4) have shown that the mineral composition of exuded maize sap depended to some extent on the fertilizer treatment. If the nutrients added in the experiments reported here were taken up by the roots and transported through the tracheal tubes, it is evident that the pathogen was growing in a medium the composition of which varied with that of the nutrient solution. If there is a difference in the composition of tracheal sap in seedlings grown under different nutrient treatments, it might change the growth rate of the bacteria or encourage the differential development of strains that vary in pathogenicity.

It is possible that competition between host and pathogen for the available nutrients may be a factor governing the severity of the disease. When the element in question is present in low concentration, the normal processes of the host cell may utilize it to such an extent as to leave an actual deficiency of it as far as the pathogen is concerned. As the supply of the element increases, the competition between host and pathogen may develop sufficiently to overcome any resistance the host cell might normally have possessed. With high-phosphorus and high-potassium nutrition, a dynamic equilibrium apparently exists between host and pathogen. With high-nitrogen nutrition, however, the pathogen was able to kill the host.

It is of interest to note the response shown by potassium-deficient seedlings to the disease. The infected seedlings developed a pronounced chlorosis, a condition not shown by the healthy seedlings. Moreover, the disease was more severe in seedlings deficient in potassium than in those supplied with low concentrations of potassium. This difference in severity may possibly be explained as follows: The pathogen may have a low potassium requirement and may cause considerable injury when inoculated into seedlings weakened by a deficiency of potassium. However, with the addition of small amounts of potassium, the seedlings start normal growth, and in so doing may become more resistant.

SUMMARY

The influence of nitrogen, phosphorus, and potassium nutrition on the reaction of sweet-corn seedlings to *Phytomonas stewarti* was studied. Severity of infection was noted 10 days after inoculation.

Seedlings dwarfed by high concentrations of nitrogen, phosphorus,

or potassium were more severely infected than those grown at concentrations more conducive to rapid growth. Seedlings deficient in either nitrogen or phosphorus were only slightly infected, whereas potassium-deficient seedlings were severely infected.

Seedlings supplied with a nitrogen-deficient solution developed small necrotic lesions but little or no wilting of invaded leaves. With high nitrogen, wilting was so intense that about one-half of the seedlings died within 2 weeks after inoculation.

At low phosphorus levels, infection was characterized by the formation of definite necrotic lesions, whereas at high levels infection was manifested not only by necrotic lesions but also by a dwarfing of the seedlings and a general wilting of the invaded leaves. None of the seedlings died as a result of infection.

In the potassium-deficient seedlings, infection was more severe than in seedlings receiving small amounts of potassium. The potassium level had no effect on the type of necrotic lesions formed, but high levels favored the wilting condition of invaded leaves.

It is evident, therefore, that mineral nutrition exerts some influence on the host-pathogen complex of bacterial wilt of maize. The exact changes brought about in this complex by nutrition have yet to be ascertained.

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"ROOT-PRESSURE"—AN UNAPPRECIATED FORCE IN SAP MOVEMENT[1]

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The long and tortuous history of our knowledge—or better our observations and theories—on the movement of sap in plants is familiar to most botanists. From the time of Stephen Hales, 200 years ago, up to the middle of the 19th century, the movement of water through the plant was supposed to be brought about primarily through the activity of living cells, either by the agency of unidentified tissues of root and stem, as Hales believed (1727), or the medullary rays, as suggested by Knight (1801). The "drawing power" of the leaves was recognized by both Hales and Knight, but, since it was well known that a suction pump would not lift water higher than about 30 feet, this was considered of secondary importance. Beginning about 1860, along with the rise of mechanistic theories in other fields, evidence began to accumulate which indicated that living cells might not be necessary for the rise of sap. This changing viewpoint found strong support in Molisch's demonstration (1902) of the traumatic nature of many of the bleeding phenomena upon which Hales' vital theory rested and finally culminated in the development of the Dixon-Askenazy cohesion theory of sap movement (Dixon and Joly, 1895; Askenazy, 1895). This theory, which takes account of the enormous suctions developed at evaporating leaf surfaces and of the fact that in capillary tubes water possesses a great tensile strength capable of transmitting these suctions through a plant stem to the soil, seemed to deal with forces more nearly commensurate with the needs of tall

[1] Paper presented at the Indianapolis meeting of the American Association for the Advancement of Science, December, 1937. The American Association prize was awarded to Dr. White for his noteworthy contribution to science presented at the annual meeting.—EDITOR.

trees than had those demonstrated by Hales and his successors. The result was that most plant physiologists completely abandoned the vital theories in favor of the mechanical ones. In spite of the objections of Priestley (1935), Ursprung (1906), Heyl (1933), and others, that is approximately where the situation rests today.

The cohesion theory certainly has some very serious flaws which are rather well outlined in Priestley's paper of two years ago (1935). It has been accepted not so much because of its freedom from objections as because of the inadequacy of all other theories. The only real contender—Hales' old "root-pressure" scheme—was rejected, first because pressures greater than the 1.4 atmospheres recorded by Hales himself had not been observed, and second because all demonstrations of root-pressure rested on experiments with decapitated and moribund plants subject to Molisch's criticism. Water must be raised in some Eucalyptus trees to a height of 350 feet, requiring either a push or a pull of at least 13 atmospheres. The maximum root-pressures observed were only of a magnitude of 1.4 atmospheres, they were transitory, and doubt even existed as to their presence in uninjured plants. Consequently, modern plant physiology texts for the most part treat root-pressure as unimportant.

Excised roots of tomato have been kept growing in vitro for a number of years (White, 1937). The roots form normally developed vascular strands, although completely immersed in a nutrient solution (fig. 1). The occurrence of strands in roots grown under these conditions has seemed rather anomalous. If, however, a continuous flow of liquid be assumed to take place through the roots, the presence of strands would be understandable. If such a current exists, it was thought these roots might offer a means of determining the reality or unreality of "root-pressure" and perhaps of measuring it under conditions free from Molisch's objections.

Means have, therefore, been devised of repeating Hales' original experiment, using single actively metabolizing tomato roots instead of his moribund grapevine stocks.

Capillary manometers were built to receive single roots. Specimens of a clone of excised tomato roots grown in continuous culture for the past 5 years were subcultured and allowed to stand for one week, to give the cut surfaces time to heal completely. Their bases

were then carefully inserted into manometers, and seals were made by means of miniature rubber hose corresponding to those used in the classic *Fuchsia* demonstration known to every student of plant physiology. The roots with their attached manometers were returned to

FIG. 1. Root of tomato (*Solanum lycopersicum* L.) (246th passage in vitro) showing the well developed vascular strand. × 90. (Photograph by J. A. Carlile.)

fresh flasks of nutrient, and their subsequent behavior observed (fig. 2). All manipulations had, of course, to be carried out aseptically and with as little trauma as possible.

The results of the first experiments were surprisingly good (White, 1936). The roots did secrete water from their bases into manometers. There does exist a unidirectional flow of liquid through these roots.

Root-pressure is not an artifact but a reality. This paper proposes to present some quantitative results of these experiments.

If roots of this kind are set up in two series of manometers, with capillaries of the same diameter, one series containing water, the other mercury, we would expect a decrease in the secretion rate under the mercury column corresponding to the 13.5:1 differential in weight between mercury and water. Figure 3 shows the result of such an

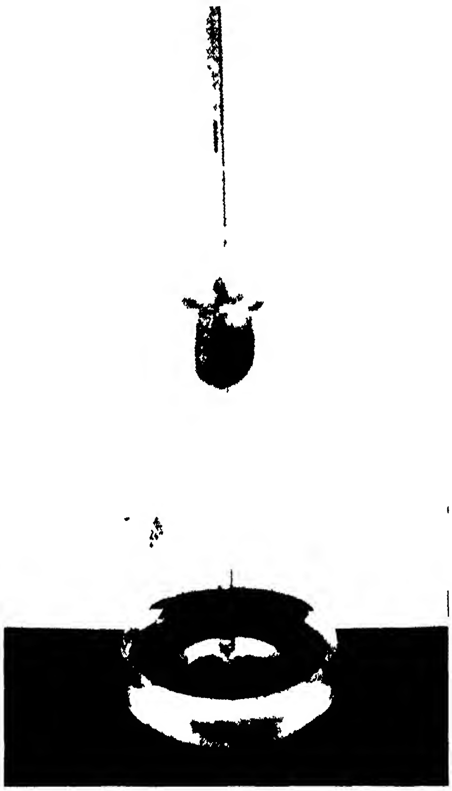

FIG. 2. Tomato root with attached manometer. (Photograph by J. A. Carlile.)

experiment using manometers 500 mm. high. Contrary to expectation, no such observable decrease occurred. Both columns rose at the same rate. The pressure differential apparently had no effect on the secretion rates. The only conclusion to be drawn is that the curves do not represent pressures at all but only volumes.

Closed manometers in which the pressure would build up rapidly with very little volume change were tried, but such manometers proved

hard to clean and did not permit the detection of leaks at the root-manometer juncture. Leveling bulbs were then resorted to. Results of an experiment with such a device are represented in figure 4. During the course of the experiment it was thrice necessary to replace or extend the manometer tube, and since the tubes were not all of the same diameter, a different scale had to be used for each. Scales were chosen, such that the slope of the curve for an hour or so before and after each change should be approximately the same. In this

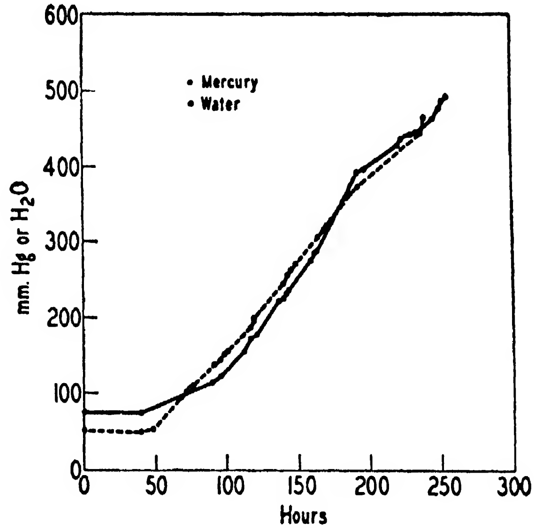

FIG. 3. Curves showing rise of liquid in two similar manometers, one filled with water, the other with mercury. The roots failed to secrete during the first 48 hours after the experiment was set up, presumably until they had recovered from the shock of manipulation. This lag does not always occur. The curves have almost identical slopes in spite of the 13.5:1 differential in weight between mercury and water.

experiment the water column rose 1790 mm. in 6 days. A leveling bulb was then attached, and an atmosphere of pressure—760 mm. Hg—was applied. Since the column continued to rise at an undiminished rate, the pressure was increased after 20 hours to 2 atm. (1520 mm. Hg). The water still continued to rise. Sufficient mercury to give another atmosphere of pressure would have carried the bulb through the ceiling of the room, so the apparatus was carefully moved to a nearby laboratory which had a stairway in it, and the bulb was carried into the room above. Unfortunately, a period of adjustment

was not allowed, as should have been done, so that when, under 3 atmospheres pressure, the column began to fall, no definite cause could be assigned for the drop. But the fact that when the pressure was reduced again to 2 atm., the column did not resume the upward course it had previously held at that pressure, indicated that a leak in the apparatus had probably been brought about in moving. The

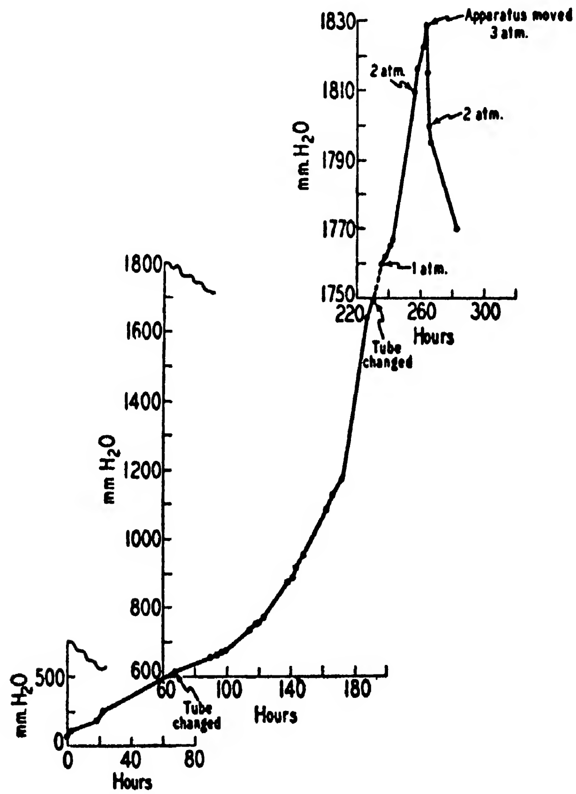

FIG. 4. A curve showing the effect of imposing pressures up to 3 atm. against the secretion pressures developed by a single tomato root.

important fact demonstrated by this experiment is that a pressure of 2 atm. was not sufficient to noticeably retard secretion of water from the uninjured base of a single root of tomato.

Since roots of this sort developed pressures in excess of 30 pounds to the square inch, it was evident that something more than the simple hose seal between glass and root would have to be devised to stand the pressures. After many trials, the assembly shown in figure 5 was

developed. The base of the manometer had a hole about 1 mm. in diameter and 15 mm. deep to receive the base of the root. Above this the opening tapered abruptly to a bore of about 0.25 mm. A centimeter back of the tip on the outside was a collar of glass. The specially made rubber connecting tube had a corresponding collar inside, with the hole about 1.5 mm. in diameter at the end which was to fit over the glass and only about 0.1 mm. in diameter where it was to enclose the root. In setting up an experiment, this tube was moistened with glycerine and the end of the manometer inserted completely through it so that the tip protruded. The base of the root was then inserted into the manometer and the rubber pushed back until it enclosed the root for a distance of about a centimeter and the glass for an equal distance. A strong linen thread was bound tightly around the rubber both over the root and over the glass. The rubber distributed the pressure so that this binding did not crush the root, while the glass collar prevented the rubber from slipping off. The whole was then enclosed in a metal clamp which had collars to press into the rubber at both ends. This effectively protected the rubber from being ruptured by the pressures applied. The manometer was then inserted into a 500 ml. Erlenmeyer flask and the upper end attached to a metal manifold. Pressure was applied to the manifold from a compressed air tank and observation begun. The manifold used took 4 manometers at a time.

In the first series of experiments with this apparatus, a gauge reading to 100 pounds per square inch was used, on the supposition that this would suffice to record any pressure obtained. Figure 6 represents the results of one experiment with such a setup. The rise of the water column was observed for 24 hours and the secretion curve, which showed a definite diurnal variation in slope,[2] plotted. Pressure of one

[2] This diurnal rhythm was observed in all experiments where readings were made at frequent enough intervals to permit its detection. It seems to be a regular characteristic of the secretion process. The roots used were not protected against the diurnal variations in temperature (24°–28°C., June, 1936) and illumination characteristic of a laboratory room with N. E. exposure. Nevertheless, since the mean daily temperatures often varied more than did the hourly temperatures within single 24-hour periods, without producing corresponding variations in secretion rate, it seems improbable that this rhythm is the result of tem-

atmosphere was applied at 4 p.m. and, since the column continued to rise, a second atmosphere was applied at 5 p.m. and the apparatus left over night. Under a pressure of 2 atm. the column rose as rapidly

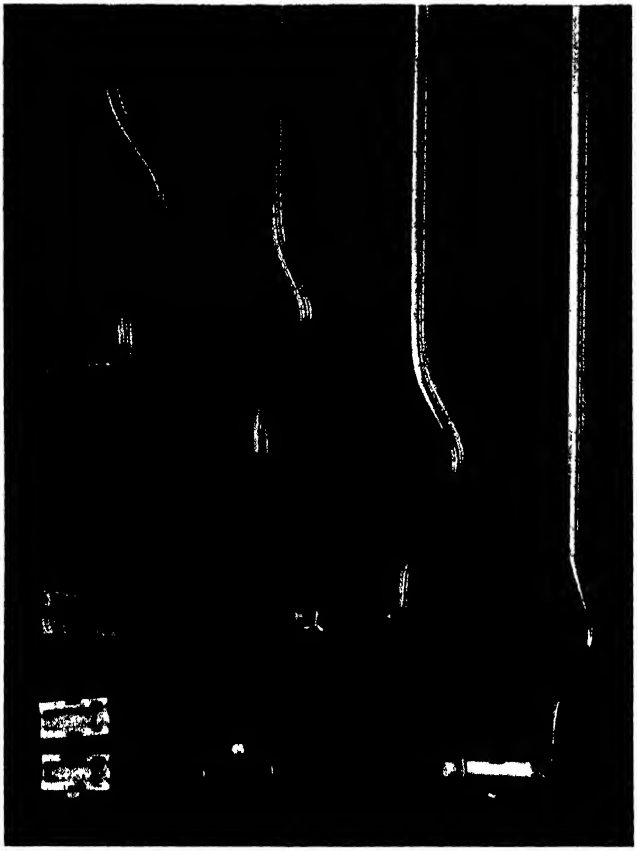

Fig. 5. Assembly, consisting of glass manometer, rubber connecting tube, and metal clamp, by which a single tomato root can be attached to a recording apparatus and its secretion force measured. (Photograph by J. A. Carlile.)

that night as it had in the corresponding period of the day before, so at 9 a.m. the pressure was increased to 3 atm., at 11 a.m. to 4 atm.,

perature variations. It is difficult, though not impossible, to imagine how an organ without chlorophyll and whose growth rate has been shown to be independent of illumination of the intensity obtained in the laboratory (White, 1937) could have this one process so markedly affected by illumination. This diurnal rhythm remains an interesting but as yet unexplained feature of the secretion process.

at 2 p.m. to 5 atm., and at 4 p.m. to 6 atm. At 7 o'clock that evening the column was still rising, and by 9 the next morning had risen an additional 110 mm., although it had been subjected to a pressure of 90 pounds per square inch over that period. As already stated, the gauge read only to 100 lbs. Another atmosphere would have given a pressure of 105 lbs., so the attempt to reach a pressure sufficient to

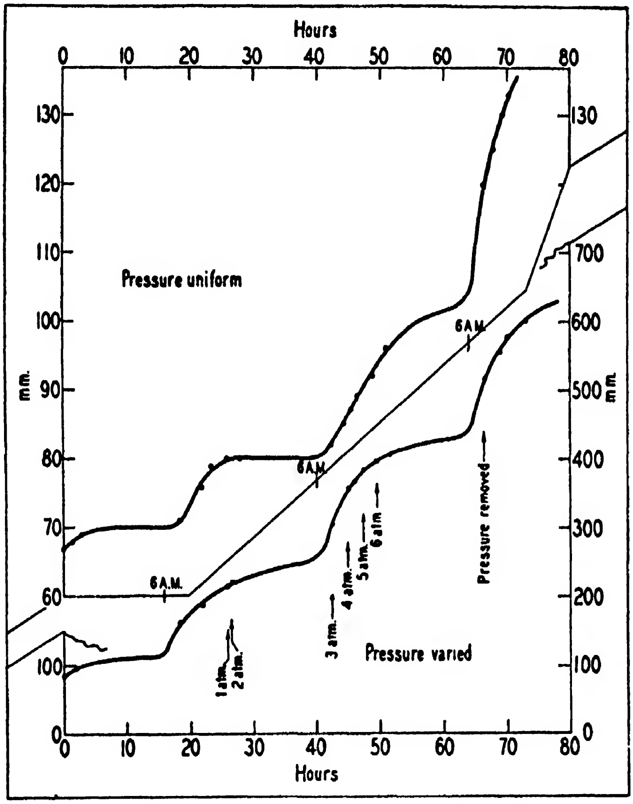

Fig. 6. Curves showing the rates of secretion of two similar tomato roots over a period of 3½ days, one with uniform (atmospheric) pressure, the other against imposed pressures up to 90 lbs./sq. in.

stop secretion had to be abandoned. The pressure was, therefore, removed and the remainder of the curve to the top of the manometer plotted. A control curve, obtained without imposed pressure, is plotted above the experimental one, for comparison.

If, now, the control curve, recorded without imposed pressure, is compared with the experimental one, obtained under pressures vary-

ing between 0 and 6 atm., they are seen to be almost exactly alike. Moreover, if the segment: 9 a.m. to noon, on the 3rd day, is compared with the corresponding segment on the 4th day, their slopes are seen to be almost identical, although the second was obtained under zero pressure and the first under 3 atm. This record represents one of 4 manometers set up in series on a single manifold. A second gave an identical curve up to the afternoon of the 3rd day when a bacterial contamination of the culture solution set in and secretion stopped abruptly. A third gave a similar curve but with a shallower slope, possibly because the root used had a smaller diameter, and the fourth developed a leak around the root which permitted air to escape. Six atmospheres pressure, 4 times the greatest value that I have found recorded elsewhere for root-pressure (the exudation pressures of Boehm, Figdor, Molisch, and MacDougal were of quite a different sort, in that they were obtained as a result of trauma) was not enough to slow down secretion to a measurable extent.

The sap movement which we are here studying is a matter of filtration through a membrane which we usually assume to be readily permeable to water, and surely equally permeable in either direction. The membrane can, therefore, be ignored in any calculation of the forces causing filtration, at least so long as movement is slow. We have been trying to balance a measured applied force against an unknown secretion force. Secretion should stop when the external and internal forces are equal, and water should flow back through the membrane—the root—when the applied force exceeds the force of secretion. Secretion should become slower and slower as the external force approaches the value of the internal one.

Six atmospheres external pressure did not bring about any observable retardation of secretion from these roots. Only one conclusion appears possible from this observation—6 atmospheres must be so small in comparison with the secretion pressure actually developed by the roots as to be quite insignificant. It is the writer's opinion that this secretion pressure can not be less than 10 atmospheres and is probably much more than that. In fact, it seems possible that it may be limited only by the osmotic value of the cells themselves. Attempts have been made to impose still higher pressures, but they have met with mechanical difficulties that have not yet been overcome.

It is interesting that so far failures have all been due to flaws in the apparatus. The roots have not failed to secrete liquid regularly against all pressures to which they have been subjected.

The old "root-pressure" theory of Hales has been disparaged by modern plant physiology texts because it did not provide sufficient force and because of the suspicion that it might be an artifact. These experiments seem to show conclusively that both objections are invalid. "Root-pressure" is certainly a very real phenomenon, going on in uninjured normally metabolizing roots and showing a striking diurnal rhythm that is reminiscent of many vital processes. The fact that a bacterial contamination will stop secretion so suddenly indicates the metabolic character of the process. And the force developed is of a magnitude not to be despised. Six atmospheres pressure is sufficient to sustain a 200 ft. water column. This is far higher than any tomato plant ever grows. Yet such a column appears to be small in comparison with what the lifting power of tomato roots is capable of sustaining. That is a force which is to be reckoned with. It has been unappreciated in the past because, before the development of the root-culture technique, only moribund and abnormally metabolizing tissues could be studied.

It is not suggested that mechanical factors, such as transpiration pull, cohesion, capillarity, etc., do not play a considerable role in sap movement. Under conditions of high transpiration they probably do account for the movement of large volumes of water through the plant. It is merely pointed out that under certain conditions—such as those prevailing in the spring when the maple sap is flowing, though leaves have not been developed—some or all of these mechanical processes cease to function. At such times root-pressure or its equivalent, stem-pressure, is quite adequate to provide for the proper functioning of even the tallest trees.

No attempt will be made at present to explain how this force is developed. It may be said, however, that we are much interested in determining if there is a diurnal rhythm in respiration rate to correspond with the observed rhythm in rate of secretion. It will take some rather delicate methods to determine that. But it is believed that we have here a phenomenon which can be studied in detail and which may throw some light on the mechanism of secretion.

SUMMARY

Experiments have shown that excised tomato roots growing in vitro secrete sap continuously and rhythmically from their proximal ends. Methods of measuring the force of this secretion have been developed. It is not retarded by opposed pressures of 90 lbs. per sq. in. The secretion force, therefore, probably greatly exceeds this value. Since a pressure of 90 lbs. per sq. in. is sufficient to raise water to a height of 200 feet, and since the existence of such secretion pressures has been demonstrated in normally metabolizing, actively growing roots, it is concluded that "root-pressure" may be a far more important factor in sap movement than has been generally conceded.

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